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Anthropogenic Disturbance of the Herpetofauna in the Northeast US : Wildlife Disease and Habitat Modification

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ANTHROPOGENIC DISTURBANCE OF THE HERPETOFAUNA IN THE NORTHEAST US: WILDLIFE DISEASE AND HABITAT MODIFICATION

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ABSTRACT

ANTHROPOGENIC DISTURBANCE OF THE HERPETOFAUNA IN THE NORTHEAST US: WILDLIFE DISEASE AND HABITAT MODIFICATION

by Paola Dolcemascolo

Herpetofauna represent some of the most striking examples of the consequences of human impact on biotic communities. They experience the full range of anthropogenically-derived stressors: habitat loss, habitat modification and degradation, pollution, collection for food and the pet trade, nuisance killings, road mortality, and disease. In this study, I examined some of the main threats faced by herpetofauna of the Northeastern United States and their implications for management of reptile and amphibians in New Jersey. I first used molecular techniques to document and assess the prevalence of two amphibian diseases, chytridiomycosis (caused by the chytrid fungus, *Batrachochytrium dendrobatidis*) and Ranavirus, throughout the state of New Jersey. While Bd does not seem to be a problem, Ranavirus was found at eleven sites in NJ. Next, I examined the first recorded occurrence of the American Green Tree frog (*Hyla cinerea*) in New Jersey to determine if its presence could be linked to a range expansion event facilitated by climate change. Toe clips were collected from both populations and partial sequences of the mitochondrial ND1 gene were used to generate a statistical parsimony network. Four haplotypes were distinguished, with all NJ haplotypes being identical to the most prevalent Delaware haplotype and the Delaware haplotypes differing

by at most one base pair. These results suggest a recent movement of Delaware frogs into NJ. Finally, I examined populations of the Diamondback terrapin (*Malaclemys terrapin*) in two urbanized locations of the species' range in order to determine the genetic and demographic health of these populations that live in such highly disturbed habitats. I used a fragment of the mitochondrial D-loop from terrapin blood samples to examine patterns of genetic diversity among populations of terrapins collected within Jamaica Bay (from Ruler's Bar Hassock and JFK airport), Hempstead Bay and Sawmill Creek Wildlife Management Area in the NJ Meadowlands. I show that the picture of the terrapin's demographic past is a complex one, possessing signs of a bottleneck, as well as recent expansion, and that genetic diversity of the mitochondrial D-loop is not severely reduced. Genetic data confirm what other studies have shown, that dispersal capabilities of terrapins are limited.

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I would like to thank my funding sources, without which it would have been much more difficult to complete my research. I received money from the National Parks Service, from the New Jersey Water Resources Research Institute and the Conserve Wildlife Matching grant program through NJ Division of Fish and Wildlife. Thanks also need to go to the many people who helped me collect field samples and gave me feedback on my work, including Brett Bragin and Drew McQuade from the NJ Meadowlands Commission, Roger Wood from the Wetlands Institute, Karena DiLeo from the Conserve Wildlife Foundation, Kim Korth and Dave Golden from NJ Division of Fish and Wildlife, Laura Francoeur from the Port Authority of NY and NJ, Holly

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DEDICATION

To all women and any who struggle to have their voices heard and strive for equality

And

To one woman in particular, Denise

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CHAPTER 1

INTRODUCTION

1.1. Herpetofaunal susceptibility to anthropogenic disturbance

Various animal and plant taxa have been affected by anthropogenic disturbance, but herpetofauna represent some of the best examples of the consequences of human impact on biotic communities. Reptiles and amphibians are particularly affected by anthropogenic disturbance because of their limited dispersal capacity; the majority of species cannot migrate long distances to avoid disturbance and are therefore forced to co-exist with humans in oftentimes sub-optimal habitats. While some species may thrive in human-modified habitats, active management is required to make sure that these species do not suffer from the myriad threats possible in such altered landscapes. Furthermore, herpetofauna, especially amphibians, also often require different microhabitats for different life stages, and so are exposed to multiple types of threats. For these reasons, reptiles and amphibians are often seen as environmental indicators, reflections of the health of the ecosystems of which they are a part. Understanding the responses of herpetofauna to the effects of human activities can provide critical information on causes of environmental degradation and the steps needed to manage that degradation.

1.2. Amphibian Disease

Perhaps no other taxonomic group better represents the effects of anthropogenic disturbance on the health of the planet than amphibians. This taxonomic group is declining around the world (Whiles *et al.* 2006; Hamer and McDonnell 2008), with an estimated one-third of amphibian species being currently threatened with extinction, according to the IUCN Red List and the Global Amphibian Assessment (GAA) (http://www.iucnredlist.org/initiatives/amphibians/analysis; Lips *et al*. 2006; Kriger and Hero 2007). This report, initiated in 2004 and updated in 2006 and 2008, contains disturbing information. The number of threatened amphibians will most likely increase in the future, as 42% of all species show declines in their numbers, while less than 1% are increasing. In 2004, habitat loss was the major threat facing amphibians, with pollution considered the second largest threat (Mann *et al*. 2009). While the latest update of the Assessment has continued to find significant roles for both habitat loss and pollution for the disappearance of amphibians, the GAA has also documented a fungal disease discovered in the late 1990s that has been sweeping through populations on all continents that are home to amphibians (Daszak *et al*. 2001; Drew *et al*. 2006; Fisher *et al*. 2009; Kerby and Storfer 2009; Van Sluys and Hero 2009). Drastic and rapid population declines and even extinctions have been ascribed to this fungus, known as *Batrachochytrium dendrobatidis* (Bd). Yet another class of emerging infectious diseases, attributed to the genus *Ranavirus* (family Iridoviridae), has been linked to dramatic

amphibian declines as well (Daszak *et al*. 1999; Gascon *et al*. 2005; Forson and Storfer 2006; St-Amour and Lesbarrères 2007; Kerby and Storfer 2009).

These declining trends are disturbing for a number of reasons. Such a staggering loss in biodiversity will undoubtedly have significant effects on ecosystems. Amphibians in particular tend to constitute a large percentage of the biomass in healthy temperate and tropical ecosystems and, as ectotherms, the energy they store in their biomass can be efficiently transferred to higher trophic levels (Whiles *et al*. 2006; Hossack *et al*. 2010). Moreover, due to their biphasic lifestyle, that energy transfer can take place between aquatic and terrestrial systems, and therefore amphibians form an essential bridge between these two systems (Davic and Hartwell 2004; Whiles *et al*. 2006). Besides being prey for other animals, amphibians are essential predators of invertebrates, keeping the population of many insect pests in check (Whiles *et al*. 2006). Some of these invertebrates are decomposers and so the presence of amphibians influences decomposition rates, which could have an effect on nutrient cycling and even carbon dynamics (Wyman 1998). Tadpoles are important grazers in aquatic ecosystems and therefore can influence patterns of primary productivity and can alter the community composition of algae (Daszak *et al*. 1999; Whiles *et al*. 2006). Finally, many amphibians, especially salamanders, construct underground burrows and therefore play a role in soil dynamics (Davic and Hartwell 2004).

Compromised ecosystems may lead to compromised ecosystem services. Unchecked insect pests could be vectors for either wildlife disease or human disease, an alteration in algal composition could alter aquatic food webs and have an effect on fish populations, and altered soil dynamics could facilitate colonization by invasive species. In addition, researchers are just now understanding that amphibians secrete compounds from their skin which may have significant beneficial effects on human health (Doyle *et al*. 2003). Finally, it has been recognized that amphibians are environmental indicators and their globally compromised situation is a direct reflection of the globally compromised situation of Earth's ecosystems (Kriger and Hero 2007).

1.3. Range Expansions and Invasion Biology

Colonization events, often the result of range expansions, are significant ecological and evolutionary processes for a number of reasons (Slatkin 1987, Le Corre and Kremer 1998, Excoffier *et al.* 2009, Sexton *et al.* 2009), and understanding the genetic consequences of those events can provide a wealth of information on the underlying mechanisms and driving forces of colonization (Johnson 1988, Ibrahim *et al.* 1996, Templeton 1998, Emerson *et al.* 2001, Ray *et al.* 2003). In particular, the genetic structure of colonizing populations can lead to a better understanding of general migration patterns of wildlife and how these are impacted by anthropogenic activities, which is fundamental to wildlife management directives (Ernest *et al.* 2003, Pearse and

Crandall 2004, Sacks *et al.* 2005, Coulon *et al.* 2006, Schwartz *et al.* 2006, Dixon *et al.* 2007, Crompton *et al.* 2008, Brown *et al.* 2009).

One particularly relevant issue to wildlife management that can be examined through the understanding of colonization events is "invasion biology" (Sexton *et al.* 2009). While movement of organisms into novel areas is not a recent phenomenon, the unprecedented rate at which animals are being introduced into naïve habitats in the modern era is most likely attributable to human activities (Hulme 2009, Pyšek and Richardson 2010). Humans now have access to virtually the entire globe and where humans go, so follow pets as well as animal and plant hitchhikers (Rahel and Olden 2008). Human modification of habitats also often facilitates the establishment of species that would otherwise not have been able to thrive in a previously undisturbed habitat (Sakai *et al.* 2001). There is a healthy debate in the ecological community as to the threats posed by non-native species (Sakai *et al.* 2001, Pyšek and Richardson 2010). Organisms that move into novel territories (non-native or invasive) can have a variety of detrimental effects on native species (Watts *et al.* 2010, Fitzpatrick *et al.* 2012). They may directly prey on native species, thereby decreasing their numbers; they may also compete with native species for resources, thereby decreasing their survival probability (Tolley *et al.* 2008, Leavitt and Fitzgerald 2009).

Moreover, a more subtle threat can be found in the potential for interbreeding between native and non-native species (Funk *et al.* 2009, Haynes *et* al. 2012). One of the metrics

used by humans in conserving biodiversity is genetic "distinctness"(Frankham 2010, Laikre *et al.* 2010, Frankham *et al.* 2012). Breeding between native species and non-native species will affect the gene pool of both species and could complicate conservation strategies, especially if the native species are threatened or endangered (Haynes *et al.* 2012). Collecting information on the colonization process that led to the introduction of the non-native species can help wildlife management agencies and researchers formulate strategies to mitigate the impacts of the non-native species and possibly prevent large-scale catastrophes from happening in the future (Leavitt and Fitzgerald 2009, Fitzpatrick *et al.* 2012). In particular, examining the genetic structure of colonizers can help determine the genetic front of colonization and therefore the potential geographic source of colonizing individuals (Pearse and Crandall 2004, Torres-Leguizamón *et al.* 2011).

Models have been predicting significant changes in the distribution of amphibians, particularly in in the central and eastern United States, linked to changes in climate (Carey 2001, Excoffier *et al.* 2009, Hutchens and DePerno 2009, Lawler *et al.* 2009, Blaustein *et al.* 2010). Changes in amphibian behavior linked to climate patterns have already been documented; in NY State, frog species are calling 10-13 days earlier over the past 100 years (Gibbs and Breisch 2001). The American Green Tree Frog (*Hyla cinerea*) has been shown to have expanded its range in the central part of its distribution, moving 110 km north of the previous northernmost population in Illinois (Tucker *et al.* 2008). This species has been shown to thrive in association with humans and may prove to be an adept invader, taking advantage of climate change. In 2011, the first occurrence

of *H. cinerea* in New Jersey was documented, and this may be a result of this species expanding its range north from Delaware (the previous northernmost limit of the eastern part of its range). The temperature in NJ has already increased an average of 2 degrees F since 1900 and winter temperatures have increased 4 degrees F since 1970. Rainfall has increased 5-10%, and the predicted 2-8 degree F increase in temperature (Union of Concerned Scientists 2007, Center for Integrative Environmental Research 2008, New Jersey Climate Adaptation Alliance 2011) will see the regional climate of southern NJ become comparable to the current climate of DE, thereby favoring the establishment of green tree frogs. Management agencies are concerned about possible hybridization with the state threatened Pine Barrens Tree Frog, as well as general ecosystem disruption if the presence of *H. cinerea* truly is due to a range expansion and establishment of this novel species in the state of New Jersey.

1.4. Turtles in Urban Environments

While amphibians may be bearing the brunt of anthropogenic disturbance when it comes to herpetofauna, turtles have certainly not escaped unscathed (Marchand and Litvaitis 2004, Conner *et al.* 2005). The life history characteristics that have allowed turtles to achieve evolutionary success over the millennia are those that are now unfortunately rendering them susceptible to human activities (Gibbons *et al.* 2001). They are long-lived, reach sexual maturity late and have overall low reproductive rates (Tucker *et al.* 2001, Baldwin *et al.* 2005); this limits their ability to rapidly respond to environmental

disturbance. In a related manner, their longevity often leads to the erroneous assumption that populations are healthy, based solely on their present numbers, and management is deemed unnecessary (Kuo and Janzen 2004). In order to avoid this assumption and circumvent the effects of turtle life history characteristics on their susceptibility to disturbance, long-term monitoring of turtle populations has been suggested (Allendorf *et al.* 2004, Alter *et al.* 2007). Besides the ongoing collection of demographic data, which can provide information on survival rates, recruitment rates and population growth/decline (Claisse *et al.* 2008, Loughry *et al.* 2013), genetic data can also be collected. Genetic data can provide invaluable information on past events, such as population bottlenecks, that could not have been detected any other way and on current processes, such as range expansions, that are difficult to detect with traditional field-based technologies, such as population bottlenecks (Cunningham *et al.* 2002, Rosenbaum *et al*. 2007, DeYoung and Honeycutt 2008). Data from demographic and genetic monitoring can then be related to environmental influences and appropriate management plans can be implemented (Allendorf *et al.* 2009, Koumoundouros *et al.* 2009, Schwartz *et al*. 2006).

Information of this nature may be especially critical for turtle populations inhabiting developed areas in order to understand how turtles and humans can successfully coexist. Evidence suggests that turtles may be successful in urbanized areas, but they face a variety of threats, such as habitat degradation, road mortality, collection for the pet trade or food, predation by subsidized predators, etc. (Marchand and Litvaitis 2004, Conner *et*

al. 2005). Only through ongoing monitoring can the severity of those threats be understood and mitigated. Understanding how to manage turtles in developed areas is necessary as more and more development encroaches on turtle habitat and turtles face limited dispersal options.

1.5. Research Questions and Dissertation Structure

The overall goal of this dissertation work was to use molecular tools and field-based approaches to address the consequences of a variety of human activities on the health of amphibian and reptile populations. The results of the studies completed as part of this dissertation are being shared with local, state and national environmental and wildlife management agencies (such as the NJ Division of Fish and Wildlife, the National Park Service and the NJ Meadowlands Commission) to inform management strategies relevant to New Jersey herpetofauna.

This dissertation is written as a series of individual manuscripts and therefore there is some necessary repetition with the Methods sections. The individual chapters address the following objectives:

In Chapter 2, entitled “Emerging Infectious Diseases in New Jersey”, I returned to the site of the first documented occurrence of *Batrachochytrium dendrobatidis* (Monsen-Collar *et al.* 2010) at the New Jersey School of Conservation and was alerted to a mass

tadpole die-off in southern NJ with characteristics similar to Ranavirus outbreaks. In this study, then, I aimed to document the occurrence and extent of these two emerging infectious diseases, chytridiomycosis and Ranavirus, in the state of New Jersey in collaboration with state and regional wildlife management agencies.

In Chapter 3, entitled "Hopping into New Territory: A Case of Amphibian Range Expansion in New Jersey", I examine the recent appearance of the American Green Tree frog, *Hyla cinerea*, in New Jersey. My aim was to determine the most likely source population for the *Hyla cinerea* that colonized New Jersey. This would help understand whether or not the presence of this species represented a range expansion, which could potentially be linked to habitat modification as a result of climate change. Because Delaware was the closest geographical location that was inhabited by green tree frogs, my hypothesis was that the *H. cinerea* in NJ originated via range expansion from Delaware. Furthermore, this study documents the current range of *Hyla cinerea* in NJ, to determine whether this species existed in an isolated pocket or if it was expanding throughout the state.

The study which is the subject of Chapter 4, entitled "Anthropogenic Disturbance and Wildlife: Diamondback Terrapins in Urban Environments", had a number of objectives:

i) To examine the genetic diversity of Diamondback terrapins in urbanized areas of NY/NJ, including Jamaica Bay Wildlife Refuge, Hempstead Bay, JFK and the NJ

Meadowlands using mitochondrial DNA; I was also interested in determining whether a mitochondrial marker would possess the genetic signature of a bottleneck event due to the intense overharvesting of terrapins until the early 1900s.

ii) To examine the relatedness of Diamondback terrapins in those urbanized areas to understand potential connections between terrapin populations; I was also interested in specifically examining the relationship between the terrapins in the Meadowlands and the other populations to understand the origin of the Meadowlands terrapins population.

iii) To determine population parameters of the Meadowlands terrapins including population size, survival rates and recruitment rates.

Finally, in Chapter 5, I discuss the management implications of the studies completed.

CHAPTER 2

EMERGING INFECTIOUS DISEASES OF AMPHIBIANS IN NEW JERSEY

Abstract

While habitat loss and pollution continue to be significant threats to amphibians, emerging infectious diseases are playing a considerable role in the disappearance of these ecologically important organisms. In this study, I used molecular techniques to document and assess the prevalence of two amphibian diseases, chytridiomycosis (caused by the chytrid fungus, *Batrachochytrium dendrobatidis*) and Ranavirus, throughout the state of New Jersey. In 2009, the first known occurrence of the chytrid fungus, Bd, was documented in the state. Further sampling has not revealed any new cases of Bd in NJ. In 2011, I documented the first known occurrence of *Ranavirus* in New Jersey amphibians. Using a combination of traditional PCR and RT-PCR I showed the presence of this emerging infectious disease in both Green Frog (*Lithobates clamitans*) tadpoles and Fowler's Toad (*Anaxyrus fowleri*) tadpoles at a site within the Pinelands, in Ocean County, as well as in *L. clamitans* tadpoles at the NJ School of Conservation, in Sussex County. Nine other sites in New Jersey have also tested positive for Ranavirus infection. So far, only tadpoles seem to be affected in these areas, with dramatic symptoms being exhibited especially by Green Frog tadpoles. This disease, however, has been shown to impact both larval and adult amphibians, as well as reptiles. Additionally, my research

showed a difference between traditional and RT-PCR, with RT-PCR revealing a much higher rate of infection than traditional PCR. The Ocean County site is home to many reptile and amphibian species, including the threatened Pine Barrens Treefrog and the threatened Pine Snake. The NJ School of Conservation site is home to a wide variety of herpetofauna as well, including the Jefferson salamander, which is a species of special concern in NJ. Little is known about Ranavirus' ecology and transmission in the wild, or its potential impact on species already in decline. Further investigation of the extent of *Ranavirus* infection in New Jersey and its impact on both stable and declining species is critical.

2.1. Introduction

Perhaps no other taxonomic group better represents the loss of biodiversity and the effects of anthropogenic disturbance on the health of the planet than amphibians. This taxonomic group is declining around the world (Whiles *et al.* 2006; Hamer and McDonnell 2008), with an estimated one-third of amphibian species currently threatened with extinction, according to the IUCN Red List and the Global Amphibian Assessment (GAA) (http://www.iucnredlist.org/initiatives/amphibians/analysis; Lips *et al*. 2006; Kriger and Hero 2007). The GAA, initiated in 2004 and updated in 2006 and 2008, contains disturbing information. The number of threatened amphibians will most likely increase in the future, as 42% of all species show declines in their numbers, while less than 1% are increasing. In 2004, habitat loss was the major threat facing amphibians, with pollution considered the second largest threat (Mann *et al*. 2009). While the latest update of the Assessment documents significant roles for both habitat loss and pollution for the disappearance of amphibians, the GAA now documents a fungal disease discovered in the late 1990s that has been sweeping through populations on all continents that are home to amphibians (Daszak *et al*. 2001; Drew *et al*. 2006; Fisher *et al*. 2009; Kerby and Storfer 2009; Van Sluys and Hero 2009). Drastic and rapid population declines and even extinctions have been ascribed to this fungus, known as *Batrachochytrium dendrobatidis* (Bd). Yet another class of emerging infectious diseases, attributed to the genus *Ranavirus* (family Iridoviridae), has been linked to dramatic amphibian declines as well (Daszak *et al*. 1999; Gascon *et al*. 2005; Forson and Storfer

2006b; St-Amour and Lesbarrères 2007; Kerby and Storfer 2009). As of the 2008 GAA update, moreover, a number of declines have no clear cause, making amphibian conservation extremely difficult (http://www.iucnredlist.org/initiatives/amphibians/analysis; Schiesari *et al*. 2007).

These declining trends are disturbing for a number of reasons. Such a staggering loss in biodiversity will undoubtedly have significant effects on ecosystems. Amphibians in particular tend to constitute a large percentage of the biomass in healthy temperate and tropical ecosystems and, as ectotherms, the energy they store in their biomass can be efficiently transferred to higher trophic levels (Whiles *et al*. 2006; Hossack *et al*. 2010). Moreover, due to their biphasic lifestyle, that energy transfer can take place between aquatic and terrestrial systems, and therefore amphibians form an essential bridge between these two systems (Davic and Hartwell 2004; Whiles *et al*. 2006). Besides being prey for other animals, amphibians are essential predators of invertebrates, keeping the population of many insect pests in check (Whiles *et al*. 2006). Some of these invertebrates are decomposers and so the presence of amphibians influences decomposition rates, which could have an effect on nutrient cycling and even carbon dynamics (Wyman 1998). Tadpoles are important grazers in aquatic ecosystems and therefore can influence patterns of primary productivity and can alter the community composition of algae (Daszak *et al*. 1999; Whiles *et al*. 2006). Finally, many amphibians, especially salamanders, construct underground burrows and therefore play a role in soil dynamics (Davic and Hartwell 2004).

Compromised ecosystems may lead to compromised ecosystem services. Unchecked insect pests could be vectors for either wildlife disease or human disease, an alteration in algal composition could alter aquatic food webs and have an effect on fish populations, and altered soil dynamics could facilitate colonization by invasive species. In addition, researchers are just now understanding that amphibians secrete compounds from their skin which may have significant beneficial effects on human health (Doyle *et al*. 2003). Finally, it has been recognized that amphibians are environmental indicators and their globally compromised situation is a direct reflection of the globally compromised situation of Earth's ecosystems (Kriger and Hero 2007).

2.1.1. Emerging Amphibian Diseases

2.1.1.1. Chytridiomycosis

A number of amphibian declines in the last two decades have been attributed to the emerging infectious disease chytridiomycosis, caused by the fungus Bd. The fungus was discovered in 1998 when researchers from both Panama and Australia simultaneously noticed massive die-offs of amphibians; at the same time, a captive blue poison dart frog at the United States National Zoological Park also died of a mysterious disease (Berger *et al*. 1998; Daszak *et al*. 1999; Retallick *et al*. 2004; Gascon *et al*. 2005; Fisher *et al*. 2009). The fungus that was isolated from these events constituted a new genus and species. Like the other members of the phylum to which it belongs (the

Chytridiomycota), Bd produces motile zoospores and does not produce hyphae. Chytrids are abundant and varied, found in both soil and water and are capable of utilizing a number of different substrates, such as chitin, cellulose and keratin. Some are important to ecosystems as decomposers while others parasitize nematodes, insects, plants and algae. Bd is currently the only known chytrid that is pathogenic to vertebrates (Berger *et al*. 1999; Gascon *et al*. 2005).

Bd's motile, flagellated zoospores infect the keratinized tissue layers of amphibian skin. In adults and juveniles, Bd infection causes skin to thicken and is thought to kill its hosts via the disruption of osmoregulation and/or release of toxins; the exact mechanism of death is still unknown. Amphibian larvae can be infected, though infection is limited to mouthparts, as these are the only keratinized portion of the larval body. Infection in larvae does not lead to death (Berger *et al*. 1998; Carey *et al*. 2006; Voordouw *et al*. 2010). Analysis of Bd has failed to demonstrate the existence of resistant spores capable of tolerating extreme environmental conditions, and therefore it is thought that Bd is a relatively fragile species. Laboratory experiments have shown that Bd's optimal growth temperature is between 15 and 25 ° C. While it may be able to survive freezing for short periods of time, temperatures above 29 ° C are lethal (Piotrowski *et al*. 2004). It does not survive desiccation and water or moisture is required for transmission of zoospores (Berger *et al*. 1999; Daszak *et al*. 1999; Kriger and Hero 2007; Voordouw *et al*. 2010).

The majority of drastic and rapid population declines ascribed to Bd have occurred in upland, pristine areas (Retallick *et al*. 2004; Gascon *et al*. 2005; Lips *et al*. 2006; Whiles *et al*. 2006; Hamer and McDonnell 2008; Van Sluys and Hero 2009). In Central America, time to decline was approximately four to six months, while in Australia, populations plummeted at an even faster rate, in six to eight weeks (Gascon *et al*. 2005). Many populations were completely wiped out. Until the fungus was isolated, the declines were highly enigmatic because these areas were not seen to be suffering greatly from habitat loss and pollution. In these upland habitats, it is believed that cold temperature is the major determining factor leading to such a high impact of Bd on amphibians (Drew *et al*. 2006; Mann *et al*. 2009; Van Sluys and Hero 2009). Interestingly, Bd has also been found in lowland habitats, but the outcomes of the presence of the fungus are quite different. In these kinds of habitats, amphibians actually seem able to persist with a certain level of Bd endemic in the population (Daszak *et al*. 2001; Briggs *et al*. 2005; Carey *et al*. 2006; Mann *et al*. 2009).

2.1.1.2. Ranavirus

While in the past much more attention was given to Bd, researchers are now starting to shift their focus to the Ranaviral diseases, as their effects may be even more severe and dramatic than those caused by Bd. Ranavirus was first isolated in the mid-1960s, from *Lithobates pipiens* (Gray *et al.* 2009b) and has been found to be part of a group of double-stranded icosahedral viruses. The type species is Frog Virus-3 (FV-3) (Mao *et al*.

1997; Densmore and Green 2007), but there appear to be multiple strains within the Ranavirus group capable of infecting fish, amphibians and reptiles (Forson and Storfer 2006a; Densmore and Green 2007; St-Amour and Lesbarrères 2007). Die-offs of common toads (*Bufo bufo*) and common frogs (*Rana temporaria*) in the United Kingdom have been attributed to Ranaviral diseases (Forson and Storfer 2006a; Balseiro *et al.* 2007, Gray *et al*. 2009a) and the United States, Australia, Japan, Italy and Spain have also seen massive amphibian die-offs. Unlike the situation with Bd, die-offs are not mainly restricted to pristine areas. Die-offs also occur at a much more rapid rate, often with close to 100% mortality after less than two weeks. The virus causes skin ulcerations and massive internal hemorrhaging (St-Amour and Lesbarrères 2007) leading to abdominal edema (Une *et al.* 2009). Behaviorally, infected frogs are lethargic and swim slowly and erratically. Tadpoles are the most vulnerable amphibian life stage (Daszak *et al*. 1999), though adults are susceptible as well. Teacher *et al.* (2010) have noted that Ranaviral outbreaks seem to infect tadpoles at a higher rate in North America, while outbreaks in the UK seem to involve adults at a higher rate, though there is no clear explanation for this.

There is as of yet no clear link between population or environmental characteristics and probability of Ranavirus presence (Densmore and Green 2007). Studies point to pond-breeding amphibians being the most severely affected group, though outcomes of exposure to the virus are by no means identical among all species (Harp and Petranka 2006, St-Amour and Lesbarrères 2007). A number of researchers have noted that

Ranavirus replication is at its optimum at warmer temperatures, and this would seem to be consistent with the fact that most outbreaks are observed during summer months. Some lab studies seem to indicate that virus replication slows below 15° C (Teacher *et al.* 2010). Others, however, have postulated that it may be more likely that tadpoles are infected during the winter; detection of die-offs during the summer may merely be a result of higher visibility of amphibians and easier access to sites, increasing detection probabilities (Gray *et al.* 2009b). While there is uncertainty with regards to temperature and prevalence of Ranavirus, evidence suggests that dry conditions may be linked to disease outbreaks. During dry periods, amphibian breeding ponds dry up, leading to increased population density, and this increase in density is linked to increased infection rates (Greer *et al.* 2005). St-Amour *et al.* (2008) have found that there is a positive correlation between Ranavirus outbreaks and anthropogenic disturbance, though the exact cause of this remains unknown. Gray *et al.* (2009b) found that tadpoles inhabiting wetlands visited by cattle were almost 4 times more likely to be infected with Ranavirus compared to tadpoles in wetlands to which cattle did not have access. Lower amounts of vegetation surrounding wetlands with cattle may have caused individuals to congregate and increase risk of infection. Alternatively, the presence of agricultural pollutants in wetlands accessible to cattle may have had an effect on the presence of Ranavirus (Gray *et al.* 2009b). The effect of pollutants on the incidence of Ranavirus, though, is still unclear. As with Bd fungus, there is some evidence that low levels of chemical pollutants may actually be beneficial for amphibians in avoiding disease outbreaks; the pollutants

may interfere with the disease organisms' ability to infect hosts (Forson and Storfer 2006a, Forson and Storfer 2006b).

Though there is debate over whether or not Ranavirus (and Bd for that matter as well) is a pathogen that has recently evolved or that has always been present but has recently become virulent, the evidence is clear that both of these diseases have reached their global distribution mainly due to human activities. In parts of the United States, salamander larvae are sold as fish bait; studies have shown that many of these larvae are infected with Ranavirus (Picco *et al.* 2007, Schloegel *et al.* 2009). Ranavirus can also persist in wet sediment and water for up to two weeks (Daszak *et al.* 1999, Gray *et al.* 2009b) and boots, vehicles and equipment that are not properly cleaned can transport virus particles in sediment and water between sites. What is particularly troubling is the fact that once Ranavirus is present in ponds, the disease can persist in amphibian populations and re-infect individuals on a yearly basis (Greer *et al.* 2005, Teacher *et al.* 2009). Besides persistence in the environment, this re-infection is due to the fact that different life stages can remain sublethally infected and serve as reservoirs. Some individuals may remain asymptomatic, while others can survive symptoms; indeed, researchers have found frogs with scars that seem to have resulted from skin ulcerations (consistent with Ranavirus) (Teacher *et al.* 2009). Metamorphs leaving their natal ponds for overwintering grounds have been shown to asymptomatically carry infection (Brunner *et al.* 2004). Adults returning to breeding ponds after overwintering have been shown to carry mild infections (Brunner *et al.* 2004). Salamanders infected with *Ambystoma*

tigrinum virus (a member of the Ranavirus group closely related to FV3) may remain asymptomatic yet infective for up to 5 months from initial exposure to the virus (Brunner *et al.* 2005).

Transmission occurs either through direct contact with infected animals (for example, via cannibalism in tadpoles) or direct contact with the virus in water or moist sediment (Brunner *et al.* 2005). Cannibalism seems to greatly decrease time to death; death often occurs a mere few days after ingesting infected carcasses and 90% mortality has been observed within 5-12 days (Pearman *et al.* 2004, Harp and Petranka 2006). Because certain individuals seem to remain asymptomatic during disease outbreaks, it seems likely that there exists some form of immunity to the virus among amphibians (Greer *et al.* 2005). This immunity appears to be genetic rather than environmental (in many cases), as susceptibility to Ranavirus infection was shown to differ among clutches (Brunner *et al.* 2005). Genetic diversity in amphibian hosts seems to play an important role in Ranavirus outbreaks (Gray *et al.* 2009b), with low levels of heterozygosity corresponding to higher susceptibility to infection and slower recovery times (Pearman *et al.* 2004).

2.1.2. Study Objectives

Monsen-Collar *et al.* (2010) were the first to document the presence of the fungus *Batrachochytrium dendrobatidis* in the state of New Jersey at the NJ School of

Conservation (NJSOC). Because this is a site used by school groups and researchers who have the potential of carrying infective particles to other sites, particular attention was paid to documenting the full extent of Bd at the NJSOC. In 2011, we were alerted to a mass tadpole die-off in southern NJ (Ocean County), with characteristics similar to Ranavirus outbreaks. Given that Ranavirus was present in states neighboring NJ, we suspected this virus was the cause of the massive tadpole die-off. In collaboration with state and regional wildlife management agencies, then, this study aimed to document the occurrence and extent of the two emerging infectious amphibian diseases, chytridiomycosis and Ranavirus, in New Jersey.

2.2. Materials and Methods

Amphibians were retrieved by net or by hand in wetlands that were chosen in collaboration with the NJ Division of Fish and Wildlife Endangered and Non-game Species Program (NJDFW) biologists. Wetlands were chosen based on accessibility and proximity to NJ Calling Amphibian Monitoring Project (CAMP). For Bd, only adults and metamorphs were sampled, while for Ranavirus, the attempt was made to sample all life stages, though tadpoles were the most frequently sampled. Between June and August of 2009, 27 samples for Bd testing were collected from amphibians of various species, along with 16 water samples, at the NJ School of Conservation (Sussex County). Between 2010 and 2013, sampling for both Bd and Ranavirus occurred throughout the state (Table 2-1, Table 2-2, Table 2-3).

Bd samples - 2010			
County	**Site**	**Month**	**Number**
Sussex	NJ School of Conservation	June, July	200
Cape May	Bayshore Mall	July	11
	Railroad Ave.	July	12
	Cape May Community College	August	7
	Kimble's Beach	August	1
	Hand Ave.	August	12
	Mechanic Pond	August	29

Table 2-1. 2010 sampling information for assessment of *Batrachochytrium dendrobatidis*.

Bd samples - 2011		
County	**Month**	**Number**
Cape May	May	20
	April	63
Atlantic	April	19
Burlington	April	17
Ocean	June	40
Salem	May	56
	June	28
Passaic	April	13
	June	6
Sussex	April	19
	May	92
Morris	April	11
	June	43
Middlesex	April	27
Monmouth	April	28
Mercer	April	13
Somerset	April	11

Table 2-2. 2011 sampling information for assessment of *Batrachochytrium dendrobatidis.*

Ranavirus Samples (2011-2013)		
County	**Month**	**Number**
Ocean	May/June 2011	114
	May 2012	24
Camden	June 2012	10
Morris	April 2011	32
	June 2013	30
Sussex	April 2011	140
	June 2013	66
Passaic	June 2011	3
	June 2013	30
Monmouth	April 2011	15
Mercer	April 2011	12
Middlesex	April 2011	7
Cape May	April 2011	12
Warren	June 2013	120

Table 2-3. 2011-2013 sampling information for assessment of Ranavirus.

2.2.1. Bd Sampling

In order to sample for Bd, a cotton swab was passed over the dorsal and ventral surfaces of amphibians, focusing on the ventral surface of limbs and where the limbs meet the trunk of the body (Kriger *et al*. 2006; Monsen-Collar *et al*. 2010). Swabs were then

placed in an empty Eppendorf tube. Within 8 hours of sampling, tubes were stored at -20 ° C until they were processed. For processing, 200 µl of deionized water was added to each Eppendorf tube containing a swab. Each tube was then vortexed for approximately 30 seconds and then boiled for 10 minutes (Monsen-Collar *et al.* 2010). The liquid was then used as template DNA for RT-PCR. Primers Bd1a and Bd2a were used (Annis *et al.* 2004), along with Brilliant II SYBR Green QPCR MasterMix (Agilent Technologies) for a reaction volume of 25 µl and following the procedure outlined in Monsen-Collar *et al.* (2010). Concentrations were as follows: 1X Brilliant II SYBR Green MasterMix, 0.4 µM Forward and Reverse primers. Parameters were as follows: 95° C for 10 minutes, 40 cycles of 95° C for 45 seconds, 60° C for 30 seconds, 72° C for 30 seconds (Monsen-Collar *et al.* 2010). Appropriate positive controls consisting of Bd DNA isolate using the same boiling technique and negative controls using water in place of DNA were run for comparison for all samples tested. Samples were run in a Stratagene Model Mx 3000 P Thermalcycler (Stratagene Technologies). Samples were considered positive if they displayed an exponential increase in fluorescence comparable to the positive control.

2.2.2. Ranavirus sampling

Previous research has shown that Ranavirus DNA can be detected from toe clips of infected amphibians (St-Amour and Lesbarrères 2007). Therefore, toe clips were taken from adult and metamorph amphibians and stored in an Eppendorf tube containing Drierite desiccant to preserve Ranavirus DNA. Tubes were stored at room temperature

until ready for processing. When tadpoles were encountered, either tail clips were taken (if the individual appeared asymptomatic) or the entire tadpole was collected (if the individual was dead or manifested symptoms consistent with Ranavirus infection). Tubes of entire tadpoles were stored on ice and then frozen at -20° C until ready for extraction.

In addition to sites chosen with NJDFW, the Stafford Business Park site in Ocean County (Figure 2-1) was sampled because of the occurrence of a mass die-off of *Lithobates clamitans* tadpoles (R. Zappalorti, pers. comm.). The cause of this die-off was unknown. We visited the site on May 17, May 26 and June 16, 2011 and May 22, 2012. We used a dip net to collect any tadpoles that were either dead or dying, placed them in Eppendorf tubes and took them back to the lab to be frozen at -20° C. We also collected live, apparently healthy, *Anaxyrus fowleri* tadpoles along with pond water in Eppendorf tubes; after a period of approximately 30 seconds in the tubes, *A. fowleri* tadpoles were released back into ponds, while the water was retained and brought back to the lab to be frozen and then processed. Adult animals were treated as above, and toe clips were taken and stored in Drierite desiccant. After toe removal they were released at the point of capture. We opportunistically sampled a dead snapping turtle by removing a piece of tail and the shed skin of a northern water snake. Samples were also taken at the NJ School of Conservation (Sussex County) after encountering a die-off of *L. clamitans* tadpoles. Entire tadpoles were taken back to the lab in the manner outlined above.

Figure 2-1. Location of reported mass die-off of *L. clamitans* tadpoles, due to suspected Ranavirus outbreak.

Total genomic DNA extraction was carried out on toe clips of adult amphibians; for tadpoles, I used either the entire body of small individuals or large sections of the tail for larger individuals. Extraction was performed via proteinase K digestion and silica spin column using a QIAmp DNA Mini Kit (QIAGEN) following the manufacturer's instructions. ProK digests were left overnight in a 56° C water bath and after approximately 24 hours, tubes were placed at -20° C for at least 2 days. This seemed to increase DNA yield.

Both traditional PCR and RT-PCR were performed on samples from Stafford Business Park and the NJSOC. RT-PCR was performed on all additional Ranavirus samples from throughout the state. The same primers, MCP4 and MCP5 (Mao *et al.* 1997), were used for both types of PCR. This primer pair was designed to amplify a fragment of the gene that encodes for the major capsid protein; PCR product length was approximately 530 bp. Traditional PCR reactions were carried out in a total volume of 25 µl, with concentrations of reagents as follows: 1x PCR Buffer, 1.5 mM Magnesium Chloride, 0.2 mM dNTPs, 0.4 µM each of Forward and Reverse primer, and 0.1 units of *taq* polymerase. Two (2) µl of genomic DNA was used. PCR parameters were as follows: initial denaturation at 94° C for 2.5 minutes, followed by 25 cycles of denaturation at 94° C for 30 seconds, annealing at 50° C for 30 seconds, extension at 72° C for 30 seconds and a final extension at 72° C for 10 minutes. Reactions were run in a GeneAmp 9700 Thermocycler (Applied Biosystems). PCR products were run on a 2% 1XTAE agarose gel with SYBR Safe (Invitrogen) gel stain to check for the presence of the approximately 530-bp Ranavirus-specific amplification product. Six PCR products that were considered positive by examining a 2% agarose gel were sequenced (ABI 3130 Genetic Analyzer) and compared to sequences deposited in GENBANK known to originate from the Ranavirus major capsid protein gene. The samples showed over 99% similarity to Frog Virus-3 and thus we were confident the PCR products that had been amplified were Ranavirus.

RT-PCR reactions were carried out in a total volume of 10 µl, with 4.6 µl of genomic DNA and amounts of reagents as follows: 1X Brilliant II SYBR Green RT-PCR Master

Mix (Agilent Technologies), and 0.4 μM Forward and Reverse primers. The positive control was DNA from a *Lithobates clamitans* tadpole that had consistently tested positive for Ranavirus using traditional PCR (and which had been sequenced to confirm its identity). Amplification parameters were as follows: 10 minutes at 95° C, 40 cycles of 95° C for 45 seconds, 50° C for 30 seconds and 72° C for 30 seconds. Reactions were run on a StepOne Plus Real Time PCR System (Life Technologies). Samples were scored as positive if the fluorescence was at least as high as the positive control and the melting curve was equivalent to the melting curve of the positive control (Figure 2-7, Figure 2-8).

2.3. Results

2.3.1. *Batrachochytrium dendrobatidis*

Results from the screen for *Batrachochytrium dendrobatidis* were all negative, with the exception of 2 *Lithobates clamitans* and one water sample collected at the NJSOC in 2009. No further occurrence of the Bd fungus has since been documented at the NJSOC. When examining temperature and precipitation averages, The period from June to August 2010 was hotter and drier than the period between June to August 2009 (Table 2-4); Bd is sensitive to temperate and moisture and the choice was made to sample during cooler months at the NJSOC. Results were still negative. Furthermore, screens for Bd throughout the state remained negative as well.

Month	Precipitation (inches) in 2009	Precipitation (inches) in 2010	Mean Temperature (°F) in 2009	Mean Temperature (°F) in 2010
June	7.54	2.52	67.1	73.1
July	5.80	3.07	71.2	77.9
August	6.91	3.57	74.5	74.2

Table 2-4. Mean precipitation and temperature in Sussex County for the period June-August for years 2009 and 2010. Taken from the Office of the New Jersey State Climatologist (http://climate.rutgers.edu/stateclim/)

2.3.2. Ranavirus

We sampled six ponds at the Stafford Business Park site (Figure 2-2) and in 2011, three of the six ponds contained amphibians that tested positive for Ranavirus using traditional and RT-PCR. This is the first documented case of the disease in NJ. All adults collected at Stafford appeared healthy, with the exception of one dead Southern leopard frog (*Lithobates sphenocephalus*). The dead Southern leopard frog tested positive for Ranavirus. No other adult tested positive on any of our trips. When we first visited the site, on May 17, there were mass die-offs of *Lithobates clamitans* tadpoles (Figure 2-3.A); a number of *L. clamitans* tadpoles that were not dead were symptomatic for Ranavirus (red lesions, swelling, erratic swimming behavior) (Figure 2-3.B, 2-5.A). Other species present at the time but not symptomatic were *Hyla* spp. and *Anaxyrus*

fowleri. Traditional PCR results (Figure 2-4) indicated that 24 animals tested positive for Ranavirus in 2011. Twenty (20) of those positives were *Lithobates clamitans* tadpoles. While there were no symptomatic *A. fowleri* tadpoles on our first visit, during our second visit (May 26 2011), there appeared to be mass mortality of *A. fowleri* tadpoles and some *A. fowleri* tadpoles exhibited symptoms of Ranaviral disease. Out of six dead tadpoles collected, three tested positive for Ranavirus. *A. fowleri* tadpoles had been observed feeding on the carcasses of dead *L. clamitans* tadpoles (Figure 2-3.C).

During one of our sampling sessions at the NJSOC, a number of dead *Lithobates clamitans* tadpoles were observed in a vernal pool known to be home to a large breeding population of Wood frogs (*Lithobates sylvaticus*) and Spotted salamanders (*Ambystoma maculatum*). Tadpoles were examined and found to display symptoms consistent with Ranaviral disease (Figure 2-5. B.). Six tadpoles were collected and traditional PCR of tail clips was carried out; one sample tested positive.

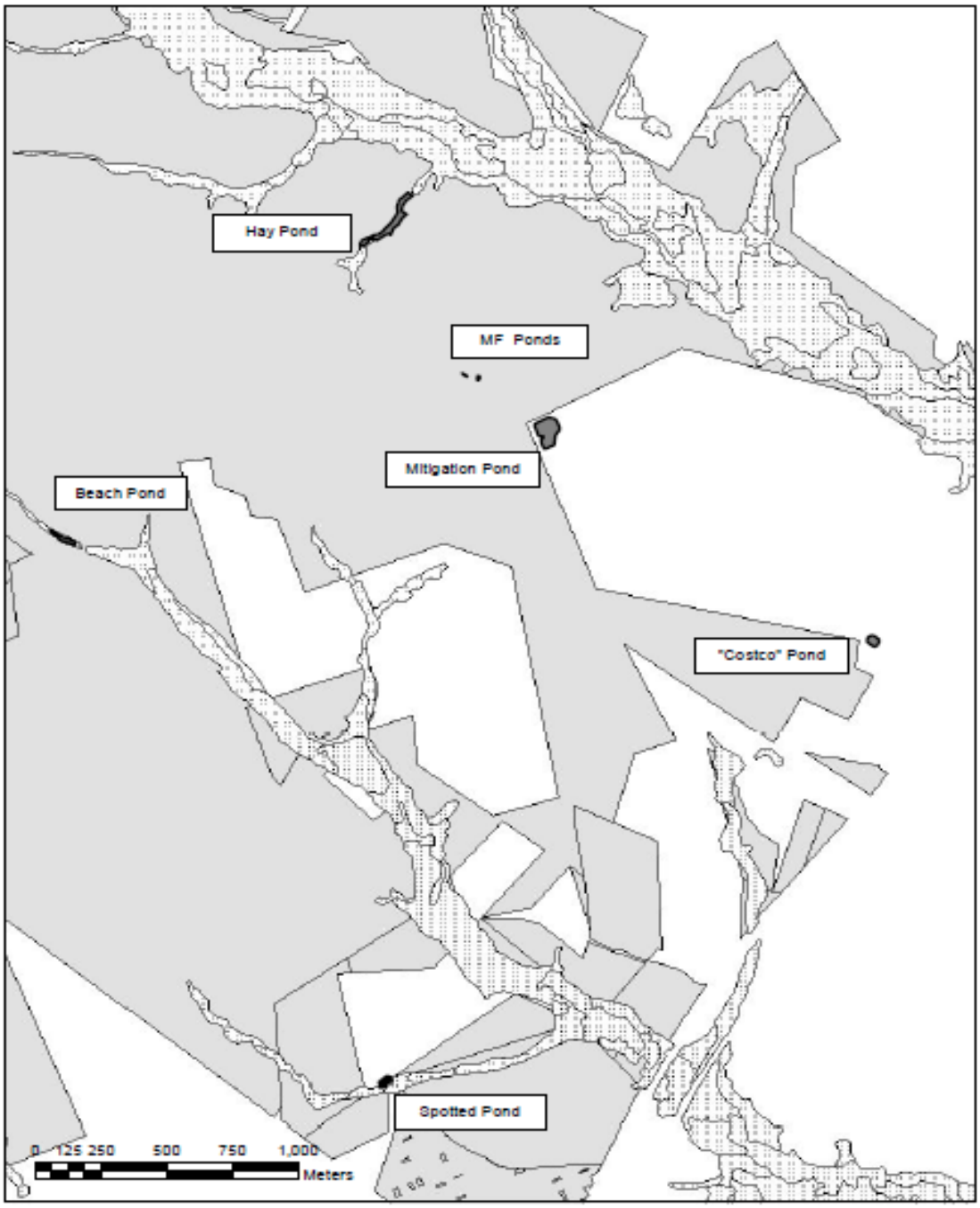


Figure 2-2. Six ponds that were sampled for Ranavirus at Stafford Business Park. In 2011, Hay Pond had 0/18 positive, Beach Pond had 0/2 positive, MF Ponds (2 ponds close enough together that they were treated as one) had 4/17 and 0/11 positive, Mitigation Pond had 26/48 positive, Costco Pond had 2/13 positive and Spotted Pond had 0/2 positive. In 2012, a total of 24 samples were collected from Hay Pond, Costco Pond and Mitigation Pond. Sixteen were positive for Ranavirus, including samples from Hay Pond and Beach Pond, which had been free of infection in 2011.

A

B

C.

Figure 2-3. Pictures taken at Stafford Business Park site to document Ranavirus outbreak. A. Mass die-off of *Lithobates clamitans* tadpoles. B. Dying *L. clamitans* tadpole exhibiting swelling and erratic swimming behavior. C. Apparently healthy *Anaxyrus fowleri* tadpoles feeding on the carcass of an *L. clamitans* tadpole.

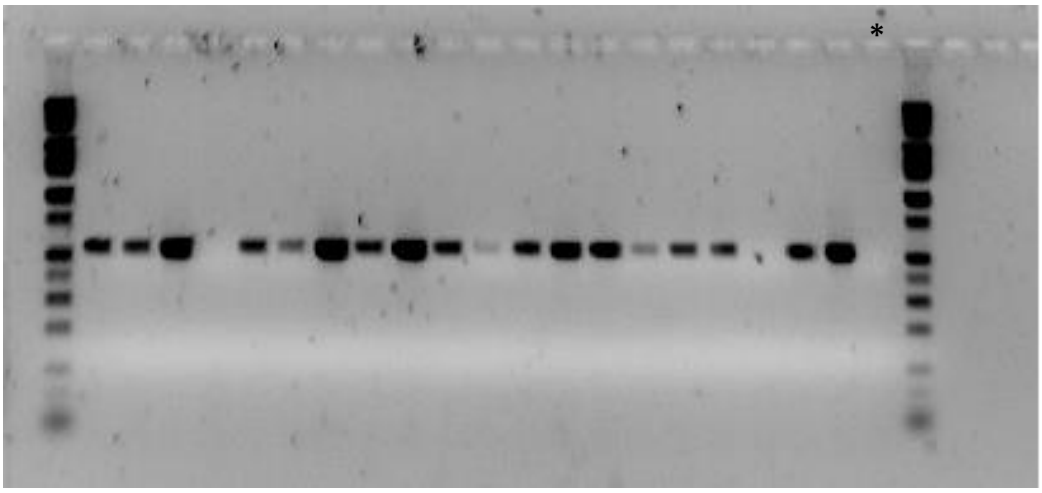


Figure 2-4. Sample 2% Agarose gel on which were run traditional PCR products. The first and last line contain DNA size standard. PCR products are slightly larger than 500 bp, making them likely candidates for the Ranavirus major capsid protein gene. Sequencing later confirmed this. The lane marked with "*" is the negative control.

2.3.2.1. Rate of Infection: Traditional PCR vs. RT-PCR

To test whether RT-PCR was more sensitive at picking up lower viral loads, we screened all the Stafford samples with RT-PCR. Thirty-two samples out of 114 tested positive with RT-PCR, as opposed to 24 that tested positive with traditional PCR. With traditional PCR, the 14 water samples from asymptomatic *A. fowleri* tadpoles collected on May 17, 2011 had all tested negative for Ranavirus. With RT-PCR, on the other hand, eight of those water samples tested positive. For samples collected in 2012, we only screened with RT-PCR and found 16 samples out of 24 collected were positive. Two ponds where

no positives had been found in 2011 were positive for Ranavirus in 2012, suggesting that the infection had spread. The six NJSOC samples were screened with RT-PCR as well and four tested positive (See Figure 2-7 for representative positive sample), as compared to one when the samples were screened using traditional PCR (Table 2-5).

Additionally, as part of a regional assessment for Ranavirus, RT-PCR has been used to document Ranavirus in eight additional sites in New Jersey, including four in Warren County, one in Morris County, two additional sites in Sussex County and one in Passaic County (See Figure 2-6 for summary map).

Site	Total samples collected	Postives with traditional PCR	Positives with RT-PCR
Stafford (2011)	114	24	32
NJSOC (2013)	6	1	4

Table 2-5. Comparison between number of positives obtained using traditional PCR and number of positives obtained using RT-PCR.

A

B

Figure 2-5. Characteristic swelling and red lesions associated with Ranavirus infection. A. *L. clamitans* from Stafford Business Park Site. B. *L. clamitans* taken from NISOC.

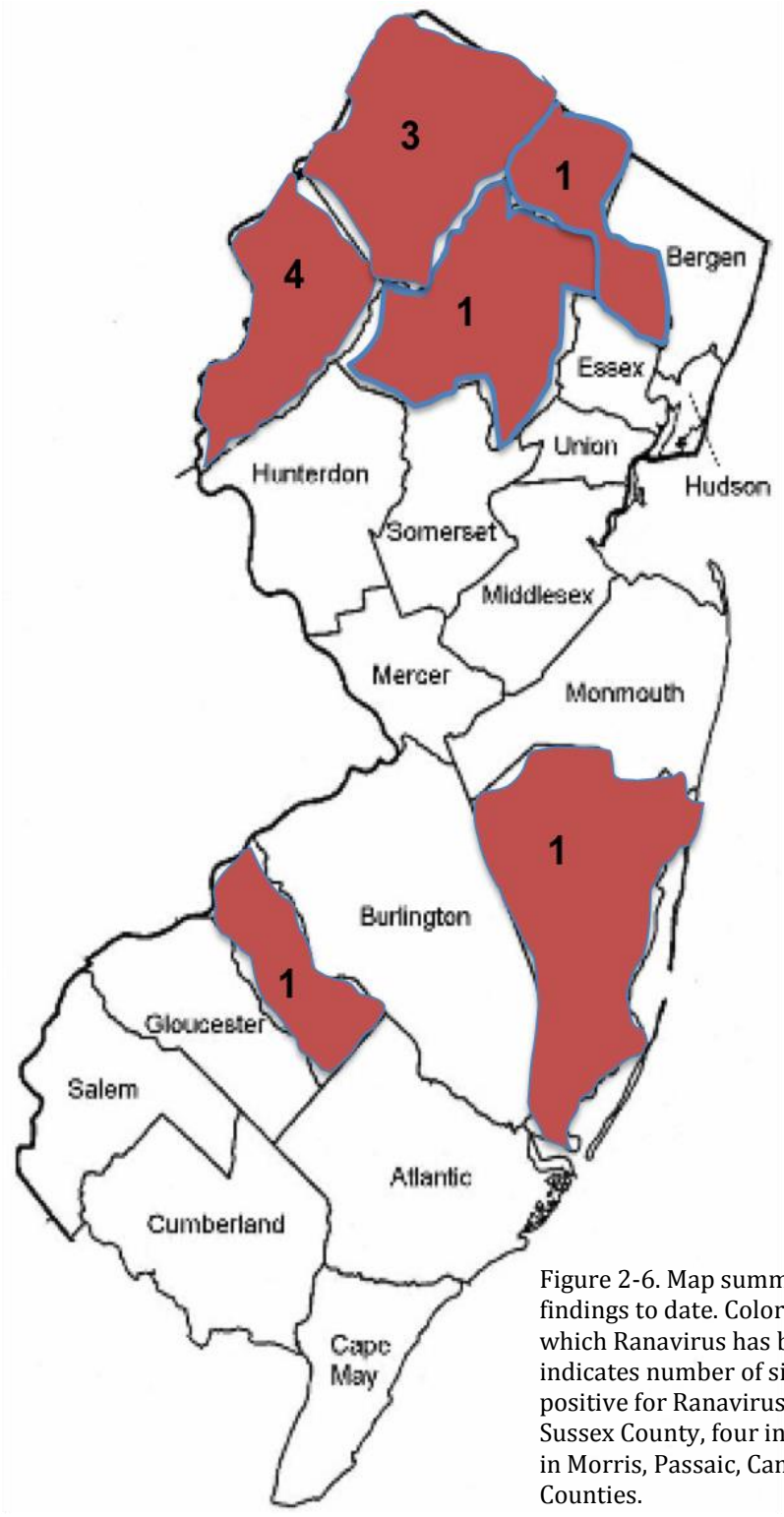
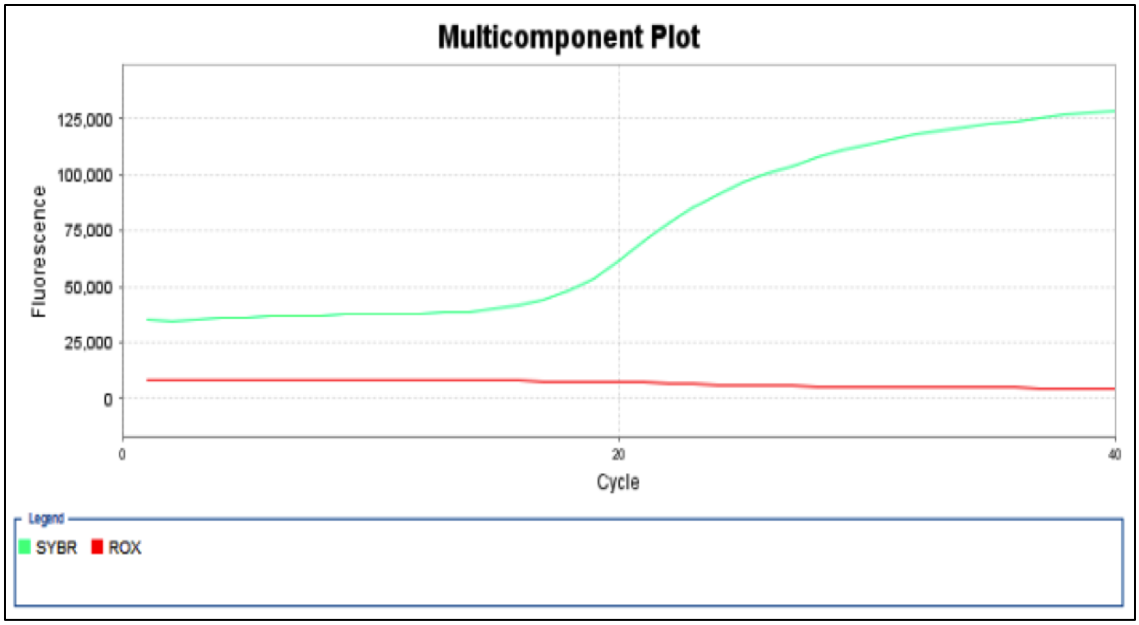
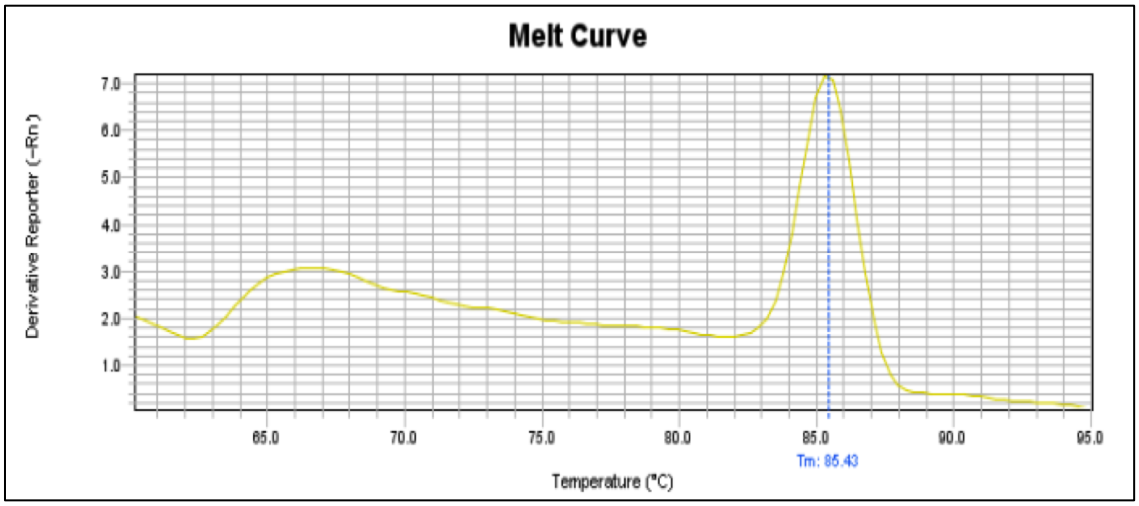


Figure 2-6. Map summarizing Ranavirus findings to date. Colored counties are those in which Ranavirus has been found. Number indicates number of sites that have tested positive for Ranavirus. To date, three sites in Sussex County, four in Warren County, 1 each in Morris, Passaic, Camden and Ocean Counties.

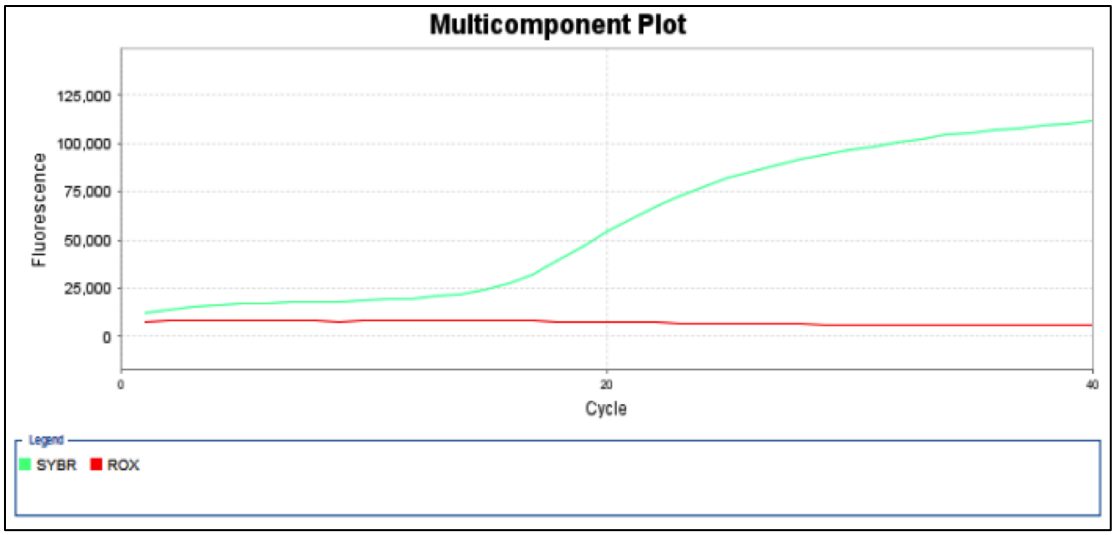


A.

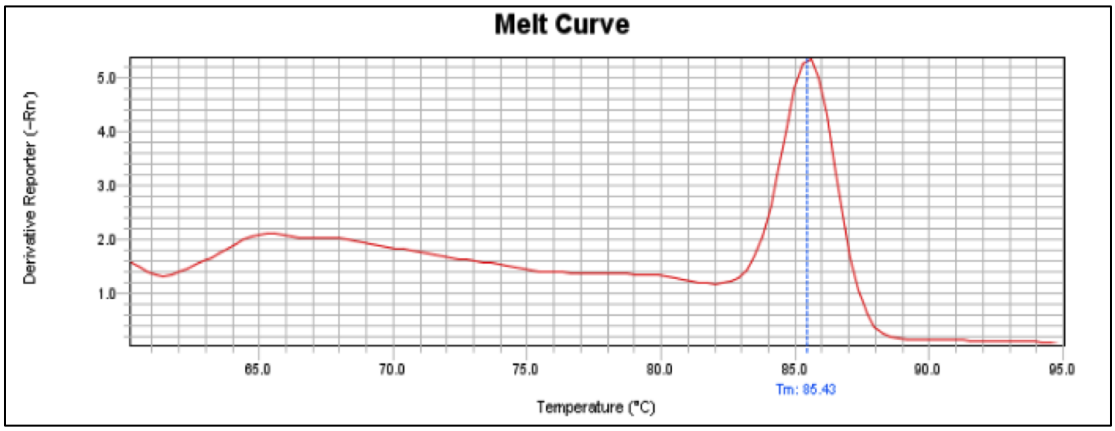


B

Figure 2-7. Fluorescence plot (A) and melting curve plot (B) for positive control (*L. clamitans* tadpole that consistently tested positive for Ranavirus major capsid protein gene, and PCR product was sequenced to confirm identity).



A.



B.

Figure 2-8. Fluorescence curve plot (A) and melting curve plot (B) of representative sample from NJSOC *L. clamitans* individual from die-off that tested positive for Ranavirus.

2.4. Discussion and Conclusions

2.4.1. *Batrachochytrium dendrobatidis*

Given the fact that Bd has caused rapid and massive die-offs in amphibian populations around the world, we were concerned with the effect the fungus would have on the amphibians at the NJSOC once it had been detected. To our surprise, however, we did not detect Bd at the School of Conservation after 2009 despite extensive sampling. This could have been due to a mass mortality event that decimated amphibian species that served as hosts to the fungus; in the absence of hosts, the fungus would have drastically decreased in abundance, possibly to the point of escaping detection. Since the site is used by school groups and is home to staff that live on-site, though, any mass mortality event involving amphibians is unlikely to have gone unnoticed. What seems more likely is that environmental conditions were unfavorable for the growth of Bd. Indeed, the precipitation decreased and the temperature increased in 2010 when compared to 2009. Bd has been shown to be a relatively fragile species of fungus, which does not tolerate heat; desiccation is lethal to the species.

2.4.2. Ranavirus

While Bd was not found in samples screened from various locations throughout NJ, an increasing number of samples have tested positive for Ranavirus since the initial

discovery of the pathogen in the state in 2011. Ponds that were uninfected at the Stafford Business Park site in 2011 were affected in 2012 and symptomatic animals were found in all ponds tested, suggesting that the infection was spreading. Locations adjacent to the Stafford Business Park site are home to breeding habitat for the threatened Pine Barrens tree frog (*Hyla andersonii*). The NJ population of this amphibian is one of three known disjunct populations and it is unique in that it is the northernmost population of this species. The disjunct nature of this population means that if the population is decimated (through a Ranavirus outbreak, for example), no ready source of migrants exists to keep the population from being extirpated. The Pine Barrens tree frog has already suffered from habitat loss and degradation and an outbreak of Ranavirus would have a significant negative impact on its long-term survival prospects in the state. The location of the Ranavirus outbreak at the NJ School of Conservation, as mentioned, was a vernal pool that is a known breeding location for obligate vernal pool breeders. Besides wood frogs and spotted salamanders, which have been documented at this site on a number of occasions, a Jefferson salamander was documented at this location at least once; Jefferson salamanders are a species of special concern in NJ. The NJ School of Conservation is within the known range of the endangered Blue-Spotted salamander (though to my knowledge none has been documented at the School of Conservation). Ranavirus at these locations is troubling for the effect it may have on these ecologically sensitive amphibian species. Additionally, Ranavirus has been documented in eight additional sites in New Jersey, suggesting this pathogen is widespread throughout the state.

Amphibians are not the only ones under threat from Ranavirus, though. Stafford Business Park is a developed site that is being managed for conservation of the threatened Northern Pine Snake (*Pituophis melanoleucus*). Ranavirus has unfortunately been documented in reptiles, oftentimes with dramatically severe symptoms and high rates of mortality. To this date, box turtles (DeVoe *et al.* 2004), gopher tortoises (Westhouse *et al.* 1996), Hermann's tortoise (Marschang *et al.* 1999), soft-shelled turtles (Zhao *et al.* 2007), leaf-tailed geckos (Marschang *et al.* 2005) and green pythons (Hyatt *et al.* 2002) have shown susceptibility to Ranavirus-like pathogens. This is disturbing for two reasons. First of all, the presence of Ranavirus at a site of known Northern Pine snake breeding habitat could put this threatened NJ reptile species at risk of further population decline. Second, though some populations may immediately die out after an outbreak of Ranavirus, while others may survive and never show signs of infection again, there are a number of populations that have been documented to become infected on a yearly basis. Re-infection by Ranavirus depends on reservoirs. Reptiles throughout NJ could serve as additional reservoirs for the virus, making re-infection of amphibian populations not only possible, but also likely.

Furthermore, this study points to the importance of using the most sensitive method possible for detecting Ranavirus, in particular in environmental samples (in the absence of significant tissue). Traditional PCR is significantly less expensive than RT-PCR, but may only be valid if the goal is to determine presence/absence in a particular location and only if tissue samples from animals at advanced stages of the disease are available.

Indeed, traditional PCR of water from asymptomatic *A. fowleri tadpoles* from the Stafford Business Park site tested negative with traditional PCR. Using RT-PCR, 8 out of 14 of those samples tested positive for Ranavirus DNA. Traditional PCR resulted in 1 out of 6 positives from the NJ School of Conservation outbreak, while RT-PCR resulted in 4 out of 6 positives. RT-PCR, therefore, is the more sensitive method and should be employed whenever possible, especially to test for Ranavirus in environmental samples (e.g. water, soil).

The World Organization for Animal Health (OIE) has listed Ranavirus, as well as chytridiomycosis, as "notifiable diseases", legally obligating countries to report their presence. This designation furthermore requires countries to monitor disease outbreaks and take steps to ensure that the virus and the fungus do not spread (Une *et al.* 2009, Teacher *et al.* 2010). Preventing the spread of these pathogenic organisms is the only way to safeguard herpetofauna, as there is currently no treatment or vaccine for wild organisms. In keeping with this directive, we have informed wildlife managers and personnel at infected sites of the necessity for decontamination. It has been shown that exposure to a 3% bleach solution for one minute was effective at inactivating Ranavirus (Bryan *et al.* 2009); cleaning equipment and containers with this disinfectant in between sampling and field work is highly recommended to prevent the spread of disease. This concentration is not lethal to amphibians and so field biologists and environmental educators handling amphibians can feel safe implementing this protocol to stem the rising tide of amphibian mortality.

CHAPTER 3

HOPPING INTO NEW TERRITORY: A CASE OF AMPHIBIAN RANGE EXPANSION IN NEW JERSEY

Abstract

In June 2011, a large population of Green tree frogs, *Hyla cinerea*, was discovered in southwestern New Jersey along the Delaware River. This was the first recorded occurrence of *H. cinerea* in NJ and represents a possible range expansion past their northern-most limit in Delaware. Subsequent reports of this species have been confirmed along the Delaware Bayshore. Northeastward range expansions by this species have been documented in Illinois and recently metamorphosed *H. cinerea* have been found 0.5 km from the nearest breeding habitat, demonstrating dispersal capacity. Management strategies concerning species that invade novel habitats will differ depending on the source of colonizing individuals. Therefore, it was my aim to determine the source of the NJ population of *H. cinerea.* Because Delaware was the closest geographical location where this species was found, my hypothesis was that the *H. cinerea* in NJ originated via range expansion from Delaware. To determine this, toe clips were collected for genetic analyses from Delaware and NJ populations and partial sequences of the mitochondrial ND1 gene were used to generate a statistical parsimony network. Four haplotypes were distinguished, with all NJ haplotypes being identical to the most prevalent Delaware

haplotype and the Delaware haplotypes differing by at most one base pair. A sequence from a Louisiana green tree frog obtained from GenBank could not be joined in the network with 95% confidence. These results indicate a recent movement of Delaware frogs into NJ. Although movement may have been human-mediated, rising temperatures are possibly favoring the persistence and establishment of these frogs in new areas. If *H. cinerea* establishes itself in New Jersey, there could be long-term impacts on native NJ species and ecosystems.

3.1. Introduction

3.1.1. Colonization Events

Colonization events are significant ecological and evolutionary processes for a number of reasons (Slatkin 1987, Le Corre and Kremer 1998, Excoffier *et al.* 2009, Sexton *et al.* 2009), and understanding the genetic consequences of those events can provide a wealth of information on the underlying mechanisms and driving forces of colonization (Johnson 1988, Ibrahim *et al.* 1996, Templeton 1998, Emerson *et al.* 2001, Ray *et al.* 2003). At the heart of a number of cases of speciation, for example, are colonization events, in which individuals expanded into novel territories, became isolated from their source population and began to diversify to the point of being distinct from the original population; this has occurred for Hawaiian *Drosophila*, the *Ensatina* salamander complex and Mauritian macaques (Templeton 1980, Barton and Charlesworth 1984, Carson and Templeton 1984, Lawler *et al.* 1995, Wake 1997, Irwin *et al.* 2001). All too often, researchers are limited to extrapolating the details of speciation events, as they occurred in the distant past. While hypotheses regarding speciation are typically based on sound evidence and well-developed models, direct confirmation is usually lacking. However, if the colonization even is witnessed close to its inception point, it can provide researchers with a more direct view of the process of speciation, especially with regards to its initial stages. In this way, scientists can observe speciation "in action" and confirm models and theories. While understanding speciation is more theoretical and perhaps

beyond the scope of wildlife management, understanding migration patterns of wildlife and how these are impacted by anthropogenic activities is well within the purview of wildlife management directives (Sacks *et al.* 2005, Coulon *et al.* 2006, Schwartz *et al.* 2006). Examining the genetic structure of colonizing populations can shed light on how colonization happened, and therefore what kind of movement patterns are being exhibited by colonizing populations (Ernest *et al.* 2003, Pearse and Crandall 2004, Sacks *et al.* 2005, Dixon *et al.* 2007, Crompton *et al.* 2008, Brown *et al.* 2009). These can be related to biotic and abiotic factors to potentially understand underlying reasons for species movement (Coulon *et al.* 2006).

One particularly relevant issue to wildlife management that can be examined through the understanding of colonization events is "invasion biology" (Sexton *et al.* 2009). While movement of organisms into novel areas is not a recent phenomenon, the unprecedented rate at which animals are being introduced into naïve habitats in the modern era is most likely attributable to human activities (Hulme 2009, Pyšek and Richardson 2010). Humans now have access to virtually the entire globe and where humans go, so follow pets as well as animal and plant hitchhikers (Rahel and Olden 2008). Human modification of habitats also often facilitates the establishment of species that would otherwise not have been able to thrive in a previously undisturbed habitat (Sakai *et al.* 2001). There is a healthy debate in the ecological community as to the threats posed by non-native species (Sakai *et al.* 2001, Pyšek and Richardson 2010) and, indeed, the literature tends to distinguish between non-native species, or those that simply did not

evolve in a particular area, vs. invasive species, which are species that grow aggressively and often (though not necessarily always) have detrimental impacts on native species (Colautti and MacIsaac 2004). There is agreement on the characteristics of species that invade or simply colonize new habitats: they are usually generalists and can tolerate (and even thrive) in heavily degraded habitats (Sakai *et al.* 2001, Pyšek and Richardson 2010, Thomas 2010). While the exact nature of the threat from any particular invasive species may be questioned, there are certainly cases where invasions have proven to have negative consequences on the ecosystems being invaded (Funk *et al.* 2009, Laikre *et al.* 2010, Pyšek and Richardson 2010). Indeed, organizations such as the USDA, the IUCN and the ISSG have compiled a ranking of the most invasive species in the world and various task forces have been created to deal with the threat from these invasive species, which count among their numbers amphibians and reptiles (www.issg.org).

Organisms that move into novel territories (non-native or invasive) can have a variety of detrimental effects on native species (Watts *et al.* 2010, Fitzpatrick *et al.* 2012). They may directly prey on native species, thereby decreasing their numbers; they may also compete with native species for resources, thereby decreasing their survival probability (Tolley *et al.* 2008, Leavitt and Fitzgerald 2009). Moreover, a more subtle threat can be found in the potential for interbreeding between native and non-native species (Funk *et al.* 2009, Haynes *et* al. 2012). One of the metrics used by humans in conserving biodiversity is genetic "distinctness"(Frankham 2010, Laikre *et al.* 2010, Frankham *et al.* 2012). Breeding between native species and non-native species will affect the gene pool

of both species and could complicate conservation strategies, especially if the native species are threatened or endangered (Haynes *et al*. 2012). Collecting information on the colonization process that led to the introduction of the non-native species can help wildlife management agencies and researchers formulate strategies to mitigate the impacts of the non-native species and possibly prevent large-scale catastrophes from happening in the future (Leavitt and Fitzgerald 2009, Fitzpatrick *et al.* 2012). In particular, examining the genetic structure of colonizers can help determine the genetic front of colonization and therefore the potential geographic source of colonizing individuals (Pearse and Crandall 2004, Torres-Leguizamón *et al.* 2011).

Traditionally, there have been two models (with variations on these two models) explaining the process of colonization of new areas, the infinite island model and the stepping stone model (Slatkin, 1985) (Figure 3-1).

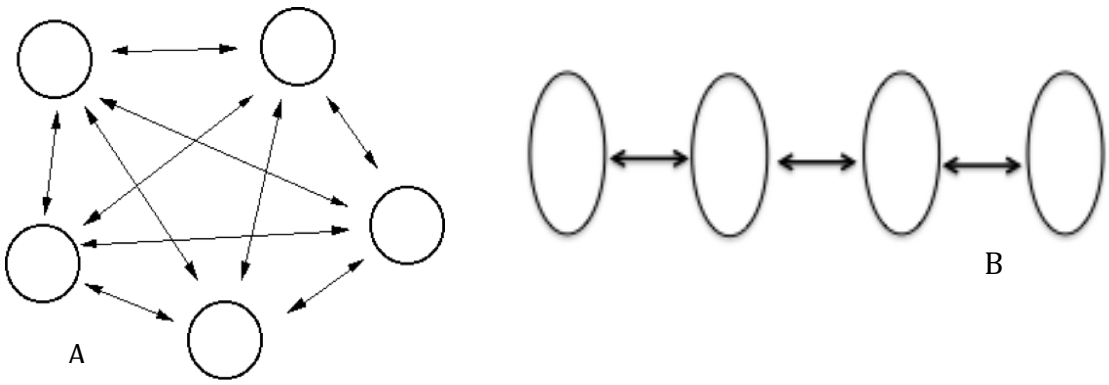


Figure 3-1. The infinite island model (A) and the stepping stone model (B) of population movement. Based on Whitlock and McCauley (1999).

In the infinite island model, a metapopulation consists of multiple demes, all of which are equally likely to exchange individuals among them. In the stepping stone model, demes are much more likely to exchange individuals with those demes immediately adjacent to them (Slatkin 1987). In the former case, the founder effect is potentially weak, since genetic diversity can be maintained, as genes are randomly shuffled around. In the stepping stone model, there is the potential for a very strong founder effect, as each deme contains only a subset of the genes of the adjacent deme (Johnson 1988, Austerlitz *et al.* 1997, Excoffier *et al.* 2007). Indeed, migration is more constrained than in the infinite island model. Biologically speaking, the stepping stone model is more likely to happen in naturally occurring populations that disperse (Ibrahim *et al.* 1996, Le Corre and Kremer 1998). Because of the strong founder effect in this model, as one moves further away from the source population, genetic diversity decreases (Templeton 1980, Tolley *et al.* 2008, Watts *et al.* 2010, Torres-Leguizamón *et al.* 2011); this means that colonization events can be distinguished by a decrease in genetic diversity over a geographic gradient (Johnson 1988, Austerlitz *et al.* 1997, Le Corre and Kremer 1998, Klopfstein *et al.* 2006, May and Beebee 2010).

More often than not, we see the genetic effects of colonization events that happened in the distant past and which we obviously did not witness. The examination of a recent event, one which can be observed close to its inception point, can provide invaluable information on the colonization process and can further elucidate what is biologically realistic (Whitlock and McCauley 1990, Templeton 1998). Indeed, empirical evidence of

colonization events from their start can help either confirm or refute our models of population dispersal.

3.1.2. The American Green Tree Frog

As stated previously, organisms that are good colonizers tend to be generalists and able to thrive in disturbed habitats, often in close association with humans. One such organism that possesses these characteristics is the American green tree frog (*Hyla cinerea*). This is a medium-sized frog with a Snout-Vent Length (SVL) of 32-64 mm (1.25 to 2.5 inches) (Somma 2012). Ground color in *H. cinerea* can vary from a light green or yellow to an olive green or slate grey and individuals possess 2 lateral stripes that can be white or yellow. There is variation within and between populations in the morphology of the lateral stripe (Aresco 1996). They are "indiscriminate and opportunistic feeders" (Leavitt and Fitzgerald 2009) and have been documented consuming insects, snails and spiders (Freed 1980, Pham 2007). Green tree frogs are found in tidal marshes, ponds, lakes, and swamps with emergent vegetation (Pham 2007); indeed, emergent vegetation seems to be critical for this species, as males will perch on this vegetation to call during the breeding season (Gunzburger 2006). They are active from late April to September and breed during late spring to early summer (Gunzburger 2006, Pham 2007). As with many invasive species, green tree frogs are often closely associated with humans and indeed thrive in developed areas. They often use eaves of buildings as retreats and are found

around house lights foraging for insects (Somma 2012). Because of their hardiness and dietary plasticity, they are popular in the pet trade (Tucker *et al.* 2008).

The green tree frog is prevalent in the southeastern part of the United States, with the distribution in the central part of the country creeping up into Illinois (Aresco 1996). Up until 2011, the northernmost limit of *H. cinerea*'s range along the east coast was the state of Delaware. This distribution was reflected in a map published online by the US Geological Survey, which had been last updated in 2005 (Figure 3-2).

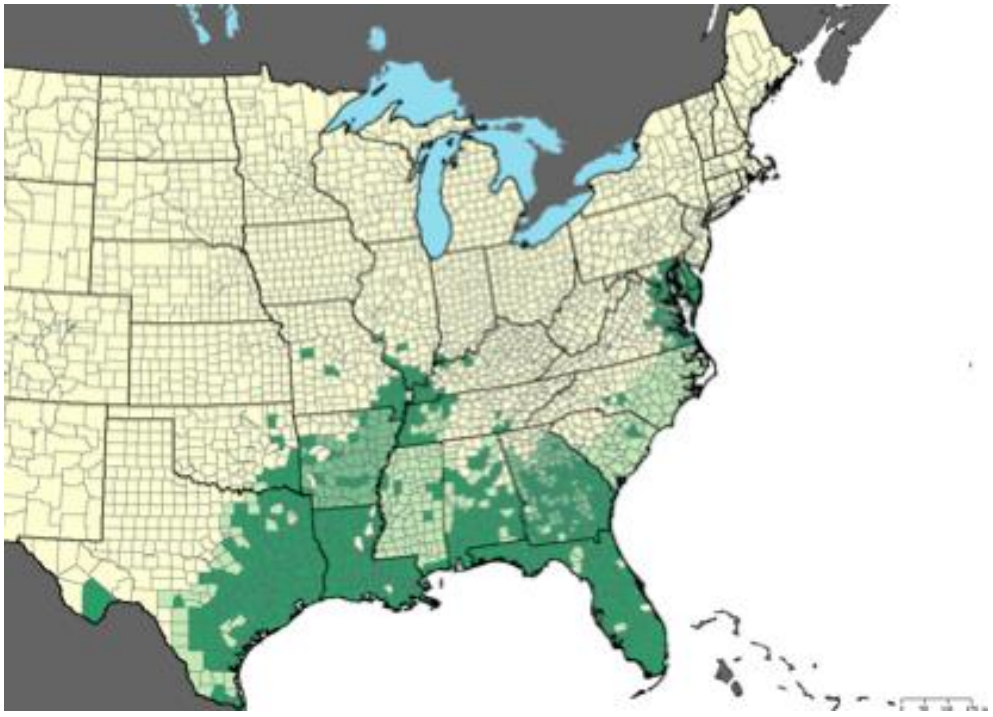


Figure 3-2. Map of *Hyla cinerea* distribution, taken from http://www.nwrc.usgs.gov/sc_armi/frogs_and_toads/hyla_cinerea.htm, which shows the geographic distribution of *Hyla cinerea* prior to 2011. Dark green = museum records, mid-level green = published records, light green = presumed presence, white = no known occurrence.

In 2011, a chorus of unidentified frogs was heard in southern NJ, in Killcohook National Wildlife Refuge (Salem County, NJ). The call was later identified as belonging to *Hyla cinerea* and upon further examination, individuals were directly observed inhabiting a freshwater tidal marsh in the refuge, which is located along the Delaware River. There were other species of frogs present and calling at the same time, including Leopard frogs (*Rana spp. nova*) and American Bullfrogs (*Lithobates catesbeianus*). The green tree frog had to my knowledge not been officially documented in the state of NJ prior to this occasion (DiLeo 2012).

Given the identification of these frogs in NJ that had not been previously recorded in the state, there were two main objectives in this study.

i). To determine the most likely source population for the *Hyla cinerea* that colonized New Jersey. This would help understand whether the presence of this species represented a range expansion or possibly a pet release that led to the establishment of a new population outside the core range. Because Delaware was the closest geographical location that was inhabited by green tree frogs, my hypothesis was that the *H. cinerea* in NJ originated via range expansion from Delaware.

ii). To document the current range of *Hyla cinerea* in NJ, to determine whether this species existed in an isolated pocket or if it was expanding throughout the state.

3.2. Materials and Methods

In 2011, *H. cinerea* individuals were collected in July from three locations, one in New Jersey, two in Delaware. On July 1, 10 individuals were collected from Killcohook National Wildlife Refuge in Salem County, New Jersey (39°37’2.50”N, 75°32’37.83”W). Animals were caught by hand at night with the use of headlamps; frogs were localized by their calls. On July 8, one frog was found dead at the base of Reedy Point Bridge in Delaware (39°33’30.70”N, 75°34’50.55”W) and a hind leg was taken as a tissue sample. On July 13, six individuals were collected from McKay House Marsh in Delaware (39°20’49.08”N, 75°32’11.81”W) (Figure 3-3); conditions of collection were the same as for Killcohook NWR. Sites were visited again for collection in 2012. On May 9, 10 frogs were collected from Killcohook NWR and on June 11, 13 frogs were collected from McKay House Marsh in Delaware, in the same manner as above. For all frogs besides the dead individual (from which a hind leg was taken), toe clips were taken before animals were release at their point of capture; scissors were sterilized with 95% ethanol in between uses.

Figure. 3-3. Locations of sampling sites for *Hyla cinerea* in Delaware and New Jersey.

Tissue samples were put into 1.5 mL Eppendorf tubes that were approximately ¾ full of Drierite desiccant and taken back to the lab. Total genomic DNA extraction was

performed via alkaline lysis and silica spin column using a QIAmp DNA Mini Kit (QIAGEN) following the manufacturer's instructions. ProK digests were left overnight in a 56° C water bath and after approximately 24 hours, tubes were placed at -20° C for at least 2 days. This seemed to increase DNA yield. For PCR, I used hylid-specific primers t-met-frog and 16S-frog (Wiens *et al.* 2005) that amplify the NADH dehydrogenase subunit 1 (ND1) of mitochondrial DNA, along with adjacent areas that include isoleucine and leucine transfer RNAs and part of the 16S ribosomal subunit 1. PCR reactions were carried out in a total volume of 25 µl, with concentrations of reagents as follows: 1x PCR Buffer, 1.5 mM Magnesium Chloride, 0.2 mM dNTPs, 0.4 µM each of Forward and Reverse primer, and 0.1 units of *taq* polymerase. Ten (10) µl of genomic DNA was used. Conditions for amplification were based on Robertson *et al.* (2009): 95° C initial denaturation for 5 minutes, 35 cycles of denaturation at 94° C for 1 minute, annealing at 50° C for 1 minute, extension at 72° C for 1 minute and a final 5 minute extension at 72° C. Reactions were then held at 4° C.

PCR reactions were cleaned for sequencing using the silica based microcentrifuge protocol of the QIAquick PCR purification kit (QIAGEN). Thirty µl of buffer EB were added to more concentrated PCR products, while 20 µl were added to more dilute products. The same primers were used in sequencing reactions on an ABI 3130 Genetic Analyzer. Big Dye terminator sequencing kits (Applied Biosystems) were used, with total reaction volumes of 20 µl. One µl of PCR product was used, with 5X sequencing buffer and a 10 µM concentration of primer. Parameters were as follows: 96° C for 1 min, 25

cycles of 96° C for 10 seconds, 50° C for 5 seconds, 60° C for 4 minutes, and a final 4° C hold. The oven temperature was preheated to 60° C while loading sequencing plates.

Sequences were checked by eye and 486 base pairs were used for the analysis. A total of twenty-nine sequences were used, 11 from New Jersey, 17 from Delaware and one from Louisiana. Mitochondrial DNA was chosen because it evolves at a faster rate than nuclear DNA (Beebee and Rowe 2007); the recent nature of the potential range expansion by *H. cinerea* precludes the use of genetic markers that evolve slowly (Van Den Bussche *et al.* 2009, Torres-Leguizamón *et al.* 2011).

Sequences were initially aligned using MUSCLE (http://www.ebi.ac.uk/Tools/services/web/toolform.ebi?tool=muscle), to be used for statistical analyses. A statistical parsimony network was drawn using the method of Templeton (1992) via the program TCS (Clement *et al.* 2000). Estimates of genetic diversity, including mean number of pairwise differences, average gene diversity, haplotype diversity and the number of polymorphic loci, were calculated using Arlequin. GenePop on the Web was used to calculate F_{st}.

3.3. Results

3.3.1. Statistical Parsimony

According to TCS, there are 4 unique haplotypes among the samples, labeled DE1, DE6, DE4 and L1 (DE = Delaware, L = Louisiana) (Figure 3-4). Twenty-six individuals had the DE1 haplotype (the most common among all individuals). Only one individual in the study had DE6, DE4 and L1, respectively. All of the New Jersey samples matched the DE1 haplotype. The Louisiana sequence (L1) was not joined to the NJ/DE haplotypes by TCS with 95% certainty, suggesting it is more distantly related.

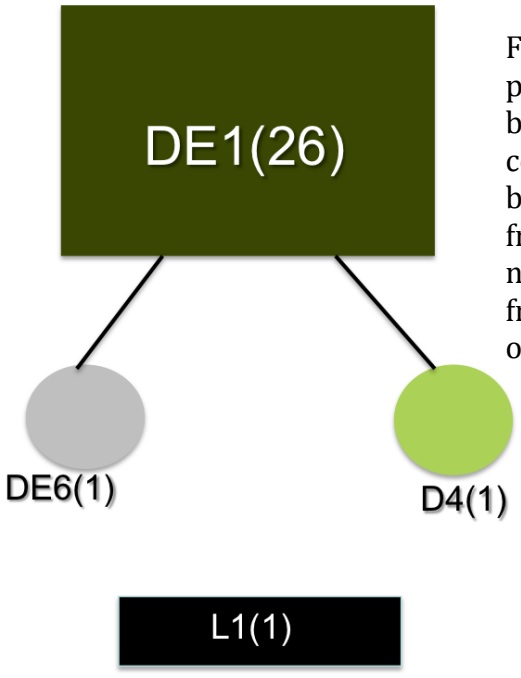


Figure 3-4. Statistical parsimony network generated by TCS. DE1 is the most common haplotype, possessed by 26 individuals. DE1 differs from DE6 and D4 by one nucleotide. L1 was obtained from GenBank and used as an outgroup.

3.3.2. Estimates of Genetic Diversity

Mean number of pairwise differences was 1.310 (+/- 0.840) and average gene diversity (haplotype diversity) over loci was 0.003 (+/- 0.002). Gene diversity indicates the probability of drawing the same two alleles at random from a sample of genes and is given by

$$1 - \sum p_i^2$$

Where p_i^2 is the probability of drawing the same allele twice

Gene diversity values range from 0 to 1. If a sample contains many alleles that are all present at similar frequencies, the gene diversity will be close to 1. If, instead, there are few alleles, with one being present at a significantly higher frequency than the rest, the value will be close to 0. Gene diversity for the *H. cinerea* sequences taken as a group was very low, indicating that there was one allele that dominated the group (and therefore a low level of diversity); indeed, TCS showed that the DE1 haplotype was the predominant haplotype (with no unique haplotypes in NJ) (Figure 3-5). Nucleotide diversity was 0.200 (+/- 0.098). This is a measure of the variation at individual nucleotide sites and in this case, the small number is indicative of the similarities between the sequences. The number of polymorphic sites, or segregating sites (S) (Wakeley 1998), was 19.

F_{st} between the NJ and DE populations was 0.025. F_{st} is a measure of the degree of genetic differentiation between subpopulations and will vary from 0 to 1 (Beebee and

Rowe 2007). The closer the value is to 1, the more genetically differentiated populations are, with a value of 1 indicating the populations are fixed for different alleles. The closer the value to 0, conversely, the less differentiated two populations are, with a value of 0 indicating no genetic differentiation at all. Little to no genetic differentiation means that there are either migrants being exchanged between the subpopulations OR that two subpopulations have split relatively recently in the past and not enough time has elapsed for them to accumulate differences (Templeton 1998, Pearse and Crandall 2004). The low F_{st} value between the NJ and DE populations indicates that there is very little differentiation between the two, which could be indicative of the NJ population having split from the DE population via a range expansion in the recent past. Insufficient time has passed for the two subpopulations to diverge genetically (Okello *et al.* 2005). Once F_{st} is obtained, the number of migrants between subpopulations can be calculated (Beebee and Rowe 2007, Carreras *et al.* 2007), and is given by:

$$N_m = \frac{1}{2}\left(\frac{1}{F_{st}} - 1\right)$$ (Slatkin 1985, Slatkin 1987, Whitlock and McCauley 1990, Templeton 1998, Pearse and Crandall 2004)

The number of migrants between the DE and NJ populations was found to be 19.5 over the course of one generation. This is a fairly high number for an amphibian. There are certain limitations, however, to this calculation (Slatkin 1987, Templeton 1998, Pearse and Crandall 2004). This approach assumes that subpopulation dynamics follow the infinite island model, which is not often the case; furthermore, it assumes that subpopulations are all roughly the same size and are all equally likely to exchange

migrants. This last point is highly unlike to occur in most biological systems. Given those limitations, though, it is often useful to calculate the number of migrants to obtain an estimate of the degree of migration occurring. The higher the number of migrants, the more genetic similarity between subpopulations.

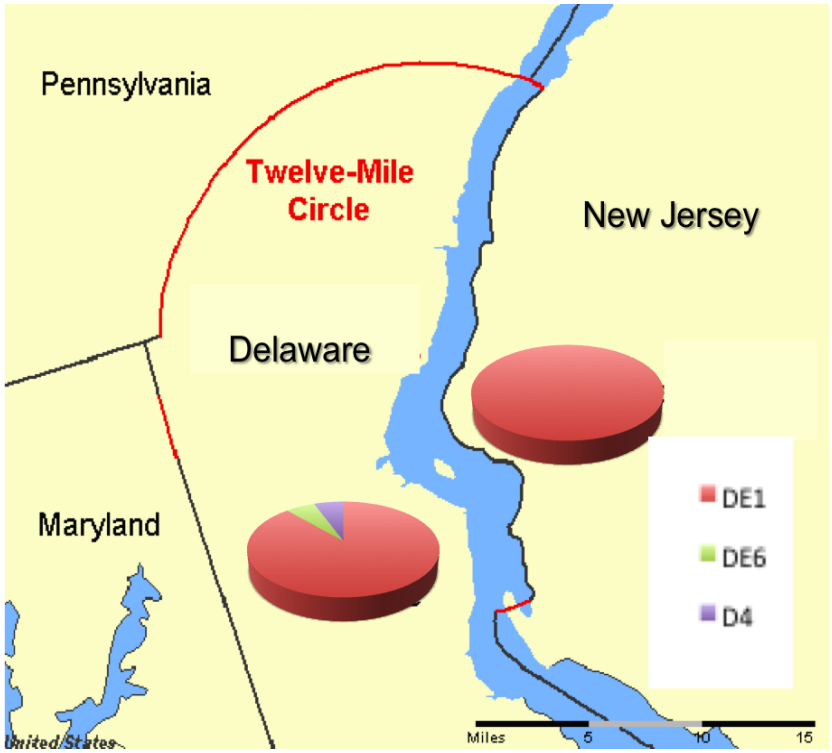


Figure 3-5. Haplotype map showing the distribution of haplotypes in NJ and DE.

Sequences were then aligned using CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to visualize distribution of the 19 polymorphic sites. Of those differences, 17 were between the Louisiana sequence (L1)

and the rest of the Delaware sequences, while only two of those differences were found in the NJ/DE group (Figure 3-6).

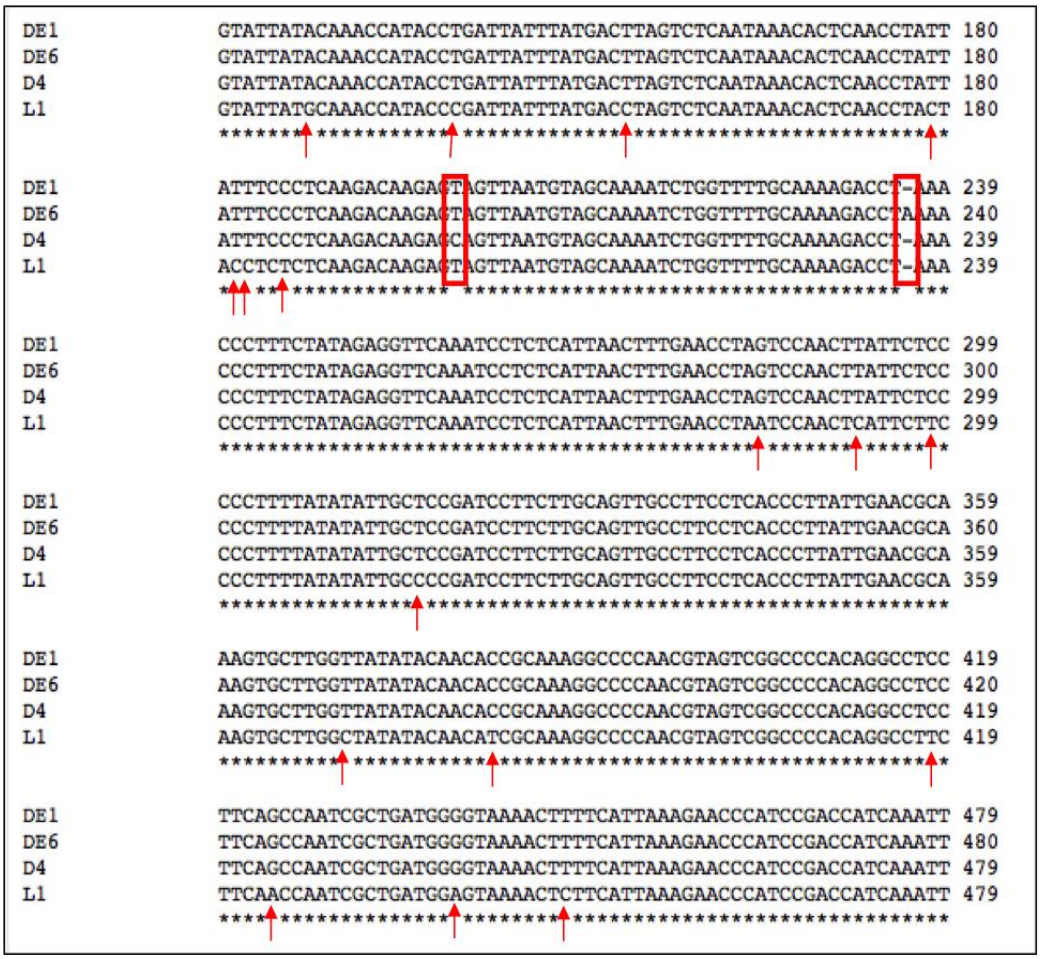


Figure 3-6. Arrows represent loci that differ between L1 and all other sequences. Rectangles represent loci that differ between DE sequences.

3.3.3. Range survey

In order to begin documenting the extent of establishment by *H. cinerea* in New Jersey, acoustic surveys were undertaken by myself, Karena DiLeo of the Conserve Wildlife Foundation of NJ and citizen scientists; the call, in fact, was put out for citizens participating in the Calling Amphibian Monitoring Project (CAMP) to add the American green tree frog to their list of possible species encountered. The presence of *H. cinerea* in Hopewell, Quinton and Greenwich (Figure 3-7) was initially reported via photograph and later confirmed in person. In Greenwich, the frogs were in a Phragmites marsh along a tributary of the Cohansey River in the vicinity of forested wetland, while in Quinton, they were found in a freshwater tidal marsh. The Hopewell case was one in which a frog fell from the second story of house; this could have been a pet release, as no large choruses were found in the vicinity. There was a report of a green tree frog in Medford, with no photographic evidence; upon further investigation, no green tree frog was found. It is thought that the frog in question may have been a juvenile gray treefrog, which is often mistaken for a green tree frog. Sightings at the PSE&G properties were made by PSE&G consultants (Karena DiLeo, personal communication).

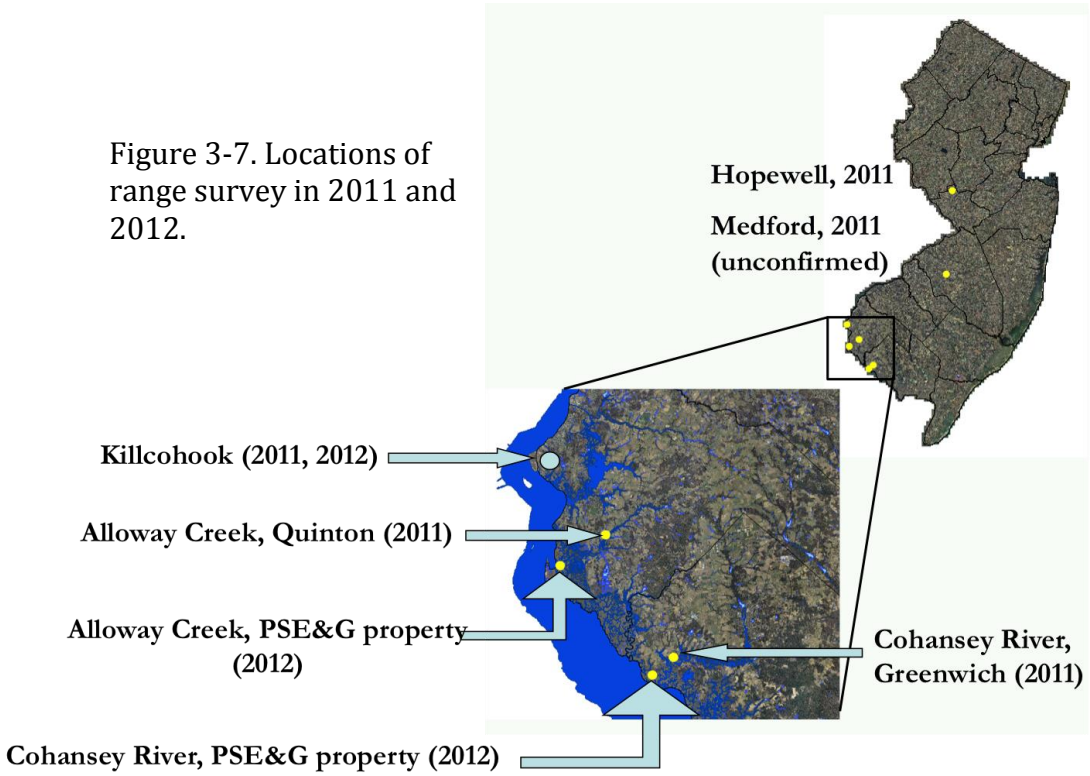


Figure 3-7. Locations of range survey in 2011 and 2012.

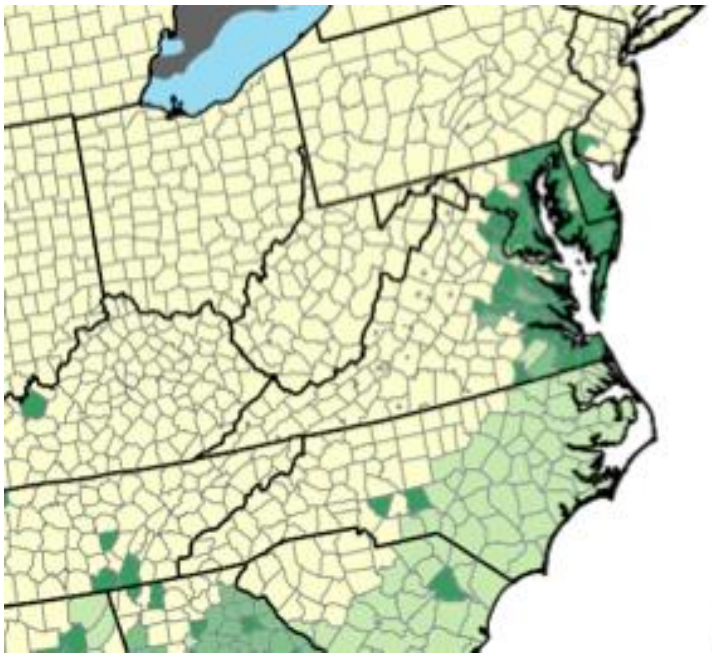


Figure 3-8. Updated map of *Hyla cinerea* distribution (created 6/18/2012), from http://www.pwrc.usgs.gov:8080/mapserver/naa/

3.4. Discussion and Conclusions

My data at least partially support the hypothesis that the NJ frogs are a result of range expansion from the northernmost limit of the species' distribution in DE. The NJ frogs show impoverished genetic diversity compared to the DE population and the only haplotype present in the NJ population is the predominant haplotype in the DE population. I used the Louisiana sequence to test whether or not that haplotype was the predominant haplotype in the species as a whole (which would be indicative of a species with low overall genetic diversity, regardless of source population), but the Louisiana sequence was not identical to either the NJ samples or the DE samples. Indeed, TCS deemed the sequence so different as to not connect it within the statistical parsimony network. The genetic impoverishment and an essentially zero F_{st} value suggest a very recent colonization event. It is likely that insufficient time has passed for the NJ population to develop enough genetic diversity to distinguish it from its founding population (all animals still exhibit the predominant haplotype from the founding population). This is a classic pattern expressed in the initial phases of colonization, due to a strong founder effect (Tolley *et al.* 2008, Excoffier *et al.* 2009, Torres-Leguizamón *et al.* 2011). Another possible hypothesis could be that the presence of the green tree frog in NJ was the result of a pet release, as these animals are used heavily in the pet trade. If this had been the case, there would have been no reason for the NJ individuals to possess the common haplotype in DE. They would have been just as likely to posses a random, unrelated haplotype or one of the minor haplotypes in the DE population. Furthermore,

pet releases of individuals are unlikely to allow for the establishment of populations, unless continued releases sustain those populations. In that case, a population would likely possess individuals with a number of different haplotypes, as random variation in store-bought animals is more likely. The pattern exhibited by the NJ frogs is indicative of a range expansion from the leading edge of the species' distribution in Delaware. The USGS has since updated its distribution map for *Hyla cinerea* as a result of this study (Figure 3-8).

This evidence for range expansion is not surprising, given the fact that models have been predicting significant changes in the distribution of amphibians, particularly in in the central and eastern United States, linked to changes in climate (Carey 2001, Excoffier *et al.* 2009, Hutchens and DePerno 2009, Lawler *et al.* 2009, Blaustein *et al.* 2010). Changes in amphibian behavior linked to climate patterns have already been documented; in NY State, frog species are calling 10-13 days earlier over the past 100 years (Gibbs and Breisch 2001). *Hyla cinerea* itself has already expanded its range in the central part of its distribution, moving 110 km north of the previous northernmost population in Illinois (Tucker *et al.* 2008). The temperature in NJ has already increased an average of 2 degrees F since 1900 and winter temperatures have increased 4 degrees F since 1970. Rainfall has increased 5-10%, and the predicted 2-8 degree F increase in temperature (Union of Concerned Scientists 2007, Center for Integrative Environmental Research 2008, New Jersey Climate Adaptation Alliance 2011) will see the regional climate of

southern NJ become comparable to the current climate of DE, thereby favoring the establishment of green tree frogs.

Genetic distinctness at the periphery of a population (and therefore in new colonizers) has been thought to be indicative of the evolutionary potential of a population (Klopfstein *et al.*2006, Gibson *et al.* 2009, Phillips *et al.* 2010). Indeed, there have been studies that show that animals that are at the periphery of their range tend to possess extreme phenotypes that are not present at the core of the range; these phenotypes have been dubbed "colonizing phenotypes", as they endow their possessors with increased abilities at colonizing new habitats (Sexton *et al.* 2009). British crickets at the edge of their range have been shown to have longer wings than crickets at the core of the range, as did speckled wood butterflies, which correlated with longer flights (Sexton *et al.* 2009); cane toads at the leading edge of their range in Australia were more likely to move and covered more distance per movement than individuals at the core of the range (Bocxlaer *et al.* 2010, Phillips *et al.* 2010).

In terms of management, then, the question becomes what to do with these colonizers. If colonizers are targeted as invasives, and therefore potentially detrimental to the ecosystems into which they are moving, extermination may be deemed necessary. Extermination, though, could potentially deplete the evolutionary potential of the species, thereby dooming them to be susceptible to climate change and other anthropogenic disturbances. Regardless of evolutionary potential, colonizers unfortunately do pose a

practical problem to humans when they cross political boundaries, such as state lines. Those animals are now new organisms within that political delineation and so the question of how to classify them and how specifically to manage them must be addressed (Tolley *et al.* 2008). Resources must be allocated in order to understand the dynamics of this new population and understand whether the range expansion was natural or man-made, like a pet release. In any case, it is advisable to monitor the process of colonization to see if the new organisms are having an effect on the ecosystem and determine if steps should be taken to mitigate their presence.

With regards to the green tree frog specifically, as of now no known negative consequences have resulted from its presence in NJ. It has demonstrated dietary plasticity outside of its native range (Leavitt and Fitzgerald 2009), but it does not seem to have impacted ecosystems in NJ. However, there is a concern. There have been documented cases of hybridization between *Hyla cinerea* and *Hyla andersonii*, the Pine Barrens tree frog, in Florida (Anderson and Moler 1986). New Jersey has a disjunct population of *H. andersonii* in southern NJ, in the vicinity of the locations that are being colonized by the green tree frog; the NJ population of *H. andersonii* is state endangered. Studies have shown that habitat degradation often leads to *H. cinerea* coming into contact and breeding with other hylid species, including *H. andersonii* and *H. gratiosa* (Anderson and Moler 1986, Aresco 1996). Anderson and Moler (1986) showed that a prolonged drought may have reduced suitable breeding habitat for both *H. cinerea* and *H. andersonii* in Florida, forcing the two species to extend their search for breeding habitat into sub-

optimal locations and to come into contact. Hybrids showed no developmental abnormalities, but were infertile (Anderson and Moler 1986). Female *H. andersonii* seem to actually prefer male *H. cinerea* (Anderson and Moler 1986) when the latter called more loudly than males of their own species. This could potentially negatively impact the endangered population of Pine Barrens tree frogs by "contaminating" the gene pool.

Future directions for this work include more thoroughly quantifying the genetic diversity in *Hyla cinerea* throughout its entire range. Our lab has requested and received museum specimens of American green tree frogs from the American Museum of Natural History to this end. We are also collaborating with the NJ Division of Fish and Wildlife to collect more tissue samples from other *H. cinerea* populations in NJ in order to determine whether the source population for these other populations is the same as that for the Killcohook population, and to continue to document the range of the species in NJ. Finally, an analysis of nuclear microsatellite markers is planned.

CHAPTER 4

ANTHROPOGENIC DISTURBANCE AND WILDLIFE: DIAMONDBACK TERRAPINS IN URBAN ENVIRONMENTS

Abstract

Herpetofauna are often limited in their ability to respond to anthropogenic disturbance mainly because of life history characteristics and limited dispersal capabilities. This means that an important survival strategy in a world of ever-increasing human development may be to live alongside humans, possibly even in highly urbanized areas. While wildlife in urbanized areas is faced with a number of threats, some organisms, such as turtles, have shown that they may be able to thrive in disturbed habitats, if managed appropriately. In order to gain more information on how some species fare in proximity to human development, I examined populations of Diamondback terrapins (*Malaclemys terrapin*) living in some of the most highly developed locations of the species' range, in NY and NJ. I used a fragment of the mitochondrial D-loop from terrapin blood samples to examine patterns of genetic diversity among populations of terrapins collected within Jamaica Bay (from Ruler's Bar Hassock and JFK airport), Hempstead Bay and Sawmill Creek Wildlife Management Area in the NJ Meadowlands. My aim was to determine the presence of a bottleneck signature within this fragment of mitochondrial DNA and correlate it to any loss of genetic diversity within the NY/NJ terrapins. Furthermore, the

origin of the Meadowlands population is unknown and therefore I aimed to shed light on how far back documentation of terrapins in the Meadowlands stretched and if these terrapins could have been recent immigrants from the NY populations. Finally, I aimed to examine demographic parameters of the Meadowlands population, since very little is known about this population. I show that the picture of the terrapin's demographic past is a complex one, possessing signs of a bottleneck, as well as recent expansion, and that genetic diversity of the mitochondrial D-loop is not severely reduced. Genetic data confirm what other studies have shown, that dispersal capabilities of terrapins are limited. Given that terrapins live in habitats often subjected to intense anthropogenic impact, as evidenced by the terrapins in this study, an understanding of their demographic and genetic characteristics is critical to the development of sound management plans for this species.

4.1. Introduction

While amphibians may be bearing the brunt of anthropogenic disturbance when it comes to herpetofauna, turtles have certainly not escaped unscathed (Marchand and Litvaitis 2004, Conner *et al.* 2005). The life history characteristics that have allowed turtles to achieve evolutionary success over the millennia are those that are now unfortunately rendering them susceptible to human activities (Gibbons *et al.* 2001). They are long-lived, reach sexual maturity late and have overall low reproductive rates (Tucker *et al.* 2001, Baldwin *et al.* 2005); this limits their ability to rapidly respond to environmental disturbance. In a related manner, their longevity often leads to the erroneous assumption that populations are healthy, based solely on their present numbers, and management is deemed unnecessary (Kuo and Janzen 2004). In order to avoid this assumption and circumvent the effects of turtle life history characteristics on their susceptibility to disturbance, long-term monitoring of turtle populations has been suggested (Allendorf *et al.* 2004, Alter *et al.* 2007). Besides the ongoing collection of demographic data, which can provide information on survival rates, recruitment rates and population growth/decline (Claisse *et al.* 2008, Loughry *et al.* 2013), genetic data can also be collected. Genetic data can provide invaluable information on past events that could not have been detected any other way and on current processes that are difficult to detect with traditional field-based technologies, such as population bottlenecks (Cunningham *et al.* 2002, Rosenbaum *et al*. 2007, DeYoung and Honeycutt 2008). Data from demographic and genetic monitoring can then be related to environmental influences and

appropriate management plans can be implemented (Allendorf *et al.* 2009, Koumoundouros *et al.* 2009, Schwartz *et al*. 2006).

Information of this nature may be especially critical for turtle populations inhabiting developed areas in order to understand how turtles and humans can successfully coexist. Evidence suggests that turtles may be successful in urbanized areas, but they face a variety of threats, such as habitat degradation, road mortality, collection for the pet trade or food, predation by subsidized predators, etc. (Marchand and Litvaitis 2004, Conner *et al.* 2005). Only through ongoing monitoring can the severity of those threats be understood and mitigated. Understanding how to manage turtles in developed areas is necessary as more and more development encroaches on turtle habitat and turtles face limited dispersal options. Furthermore, and fortunately, the appreciation for urban ecology is growing. Direct exposure to the outdoors and wildlife has been shown to improve the well being of humans (Maller *et al.* 2005, Berman *et al.* 2012) and their understanding of the importance of environmental issues (Strife and Downey 2009). People who live in urbanized areas often have limited opportunities to get outdoors and are forced to seek ecological experiences close to home (Strife and Downey 2009). The establishment and maintenance of parks and refuges that are home to wildlife, like turtles, are extremely important for these individuals. With their improved understanding of environmental issues, so grows their support for conservation programs.

4.1.1. The Diamondback terrapin

The Diamondback terrapin (*Malaclemys* terrapin) is an excellent subject for both understanding the impact that anthropogenic disturbance has had on herpetofauna, as well as understanding how turtles can survive in urbanized areas. This is the only turtle in North America that is adapted to a brackish environment (Gibbons *et al.* 2001; Tucker *et al.* 2001; Hart and Lee 2006), inhabiting salt marshes, estuarine habitats and mangroves along the eastern coast of the US, from Cape Cod to Florida and along the Gulf of Mexico, to Texas (Butler *et al.* 2006). Terrapins play important roles in their ecosystems, and there is evidence that they help to control populations of grazers, which would otherwise substantially alter estuarine environments (Silliman and Bertness 2002). Unfortunately, coastal ecosystems are some of the most heavily impacted ecosystems on the planet, putting terrapins in the direct path of human-induced habitat degradation (Baldwin *et al.* 2005; Hart and Lee 2006).

Terrapins have always had a difficult relationship with humans. Before facing their current threats, they were seen as a source of protein and harvested for food; their shells were used to fashion items such as spoons and bowls. The threat from harvesting intensified in the 1800s. A main ingredient in turtle soup, terrapins were harvested to the point of near extinction until the early 1900s (Baldwin *et al.* 2005; Brennessel 2006; Hart and Lee 2006). The decreasing popularity of turtle soup (mainly due to the difficulty of finding another crucial ingredient in the soup, sherry, during Prohibition) afforded

terrapins some protection, as did new regulations. Thanks to this kind of protection, it is thought that terrapin populations rebounded, but their numbers may now be impacted by current threats which include, besides habitat degradation, mortality in crab pots and road mortality when females migrate to nesting sites (Tucker *et al.* 2001; Avissar 2006).

Despite these threats, terrapins seem to be persisting in urbanized areas. In this study, I examined two populations of Diamondback terrapin (*Malaclemys terrapin*), found in perhaps the most urbanized locations of the species' range, Jamaica Bay, NY, and the New Jersey Hackensack Meadowlands. Although long-term monitoring has been ongoing in Jamaica Bay since 1998, very little is known about the terrapins in the NJ Meadowlands. These populations are found at the heart of an extremely developed area and present an excellent opportunity to study ways to manage urban wildlife for the benefit of ecosystem health, as well as for urban ecotourism. My aim was to understand the population structure and extent of genetic diversity of the terrapins in these locations in order to shed light on historical population processes that may have impacted the demographic and genetic health of these populations. In particular, I was looking for signals of reduced genetic diversity due to habitat fragmentation and a population bottleneck caused by severe overharvesting in the late 1800s to early 1900s. Information on the genetic and demographic characteristics of terrapin populations has been flagged as crucial for the implementation of management strategies by both the National Park Service, which manages the Jamaica Bay terrapins, as well as the NJ Meadowlands Commission, which manages the NJ Meadowlands population of terrapins.

4.1.1.1. Jamaica Bay

The population of terrapins in Jamaica Bay, NY, has been continuously monitored since 1998 to understand the threats faced by these urban-dwelling turtles (Russell Burke, pers. comm.). Jamaica Bay contains a series of small marsh islands adjacent to John F. Kennedy International airport (JFK). Marsh loss is seen as a serious problem plaguing the bay (Hartig *et al.* 2002), so much so that the National Park Service has made it a priority to attempt to mitigate this loss (Waldman 2008). The area has in general been heavily impacted by human activities over the past two centuries, with waste disposal activities having played an important role in the history of the bay (Black 1981). The terrapin monitoring program has focused on nesting females and hatchlings to gauge nesting ecology, reproductive success, nest predation rates, hatchling survival and recruitment. Nesting activity may, indeed, be shifting from Ruler's Bar island (the main marsh island in the bay, and the original focus of the monitoring program, where Jamaica Bay Wildlife Refuge is located) to the most pristine marsh left in the bay, JoCo marsh, which is located adjacent to JFK airport (Russell Burke, pers. comm.).

4.1.1.2. The New Jersey Hackensack Meadowlands

The NJ Hackensack Meadowlands is a heavily industrialized area containing wetlands, open waterways and upland areas about 6 miles west of the island of Manhattan. Like Jamaica Bay, despite its heavily urbanized location, there is a thriving wilderness and

there is much interest in visiting the Meadowlands to appreciate local ecology. What we know today as the Meadowlands, though, has undergone a significant amount of change over the years, due to both natural as well as anthropogenic causes (Quinn 1997). The events that would lead to the present-day Meadowlands began roughly 15,000 years ago, when the Wisconsin glacier retreated and left behind a series of glacial lakes, one of which was Glacial Lake Hackensack (Harshberger and Burns 1919, Maguire Group 1989, Quinn 1997). A few thousand years after the retreat of the glacier created the lake, the lake drained and in its place were left freshwater meadows composed of sedges, grasses and alders, as well as swamps of black ash (Sipple 1972, Kraus and Smith 1988). Thus began a period of significant, natural, vegetational change in the ancient Meadowlands area. Subsequent to the black ash swamps there most likely came northern bogs of larch and black spruce and following that, Atlantic white cedar swamps. It was the Atlantic white cedar swamps that were the dominant vegetational type when European colonizers arrived (Kraus and Smith 1988, Quinn 1997).

Starting in the 1600s, then, anthropogenic activity took over as the main driver of vegetational change in the Meadowlands. The Atlantic white cedar swamps were decimated and human activity drastically altered the hydrology of the area (Sipple 1972). There are reports that before the early 1800s, water flowing into Newark Bay from the Hackensack and Passaic Rivers was mostly freshwater; in a short span of time this was no longer the case (Quinn 1997). The Meadowlands were seen as a “wasteland” and intense development was undertaken to make the land suitable for agriculture and other

activities ("The Reclamation of Marshlands – A Useful Profit" 1866, "Hackensack Meadows" 1901, State of New Jersey Department of Community Affairs 1968, Quinn 1997). Dikes and drainage ditches were built, as well as dams to create millponds; freshwater was diverted into municipal water systems (Kraus and Smith 1988). Hand in hand with this alteration in hydrology came the invasion of *Phragmites*, which further altered the ecosystem of the area (Maguire Group 1989, Sipple 1972). Perhaps the most damage to the Meadowlands was done, however, in the name of eradicating mosquitoes, which were the bane of Meadowlands' life. A large number of additional dikes, tide gates and drainage ditches were built from around 1915 to 1950 in order to eliminate mosquito breeding habitat (Headlee 1911, Quinn 1997). A great storm in November of 1950 destroyed these tide gates and tidal flow was then returned to Sawmill Creek and Kingsland Creek, in the southern part of the Meadowlands, which had not seen tidal flow in over 130 years. The presence of *Phragmites* declined and *Spartina alterniflora* increased, and the area became a brackish salt marsh (Portera and MacNamara 1972, Kraus and Smith 1988).

Because of the human presence in the Meadowlands since colonial times, written records exist of the vegetation and wildlife that was encountered. Herpetofauna, including turtles, have always been abundant in the area. There are records of box turtles at the edge of Overpeck Meadows in the 1950s (Quinn 1997). Snapping turtles were collected in the Meadowlands as early as the 1890s by young boys to sell to a local hotel that served snapping turtle soup (Quinn 1997). As mentioned previously, terrapins have been used as

a food source since before colonial times. When European settlers arrived in this area, they, too, took up the practice of eating terrapins. Terrapin soup became somewhat of a delicacy in the late 1800s and early 1900s (Brennessel 2006). Terrapins fetched a hefty price and fisheries sprang up along the east coast in order to harvest these animals for a very lucrative market. Terrapin farming operations were even established (Goode 1887, Hart and Lee 2007). Because of the economic importance of terrapins, there are fairly good written records of their presence and abundance dating to colonial times. Despite this documentation, no records of terrapins in the Meadowlands seem to exist prior to the 1970s.

The absence of terrapins in this area may be attributable to two factors. The salt marshes on which terrapins are dependent seem to have been scarce in the Meadowlands prior to the 1950s (Sipple 1972, Kraus and Smith 1988, Quinn 1997) and those that were present were heavily impacted by human activities (Quinn 1997). Therefore, even if there were terrapins in the Meadowlands before the first official record of them, their numbers must have been small. In 1972-1973, the NJ Division of Fish, Game and Shellfisheries conducted a study of the marshland in the Kingsland/Sawmill marshes (which today are home to a large population of terrapins) and did not report the presence of terrapins. The first mention of the diamondback terrapin in the Meadowlands was in a 1975 report by the Hackensack Meadowlands Development Commission ("Wetland Bio-Zones of the Hackensack Meadowlands: An Inventory" 1975). Since that first mention the New Jersey Meadowlands Commission (NJMC) has seen an increase in the number of

terrapins present. In 2009, the NJMC began a mark-recapture study of the terrapins in the Sawmill Creek Wildlife Management area, which contained one of the healthiest examples of brackish marsh in the Meadowlands area, and therefore contained suitable habitat for terrapins (Brett Bragin pers. comm.). In contrast to the Jamaica Bay monitoring program, both males and females in the Meadowlands were captured and so a more thorough understanding of Diamondback terrapin urban ecology can be obtained from the study of this unique, under-studied population.

4.1.2. Study Objectives and Hypotheses

This study had a number of objectives:

i) To examine the genetic diversity of Diamondback terrapins in urbanized areas of NY/NJ, including Jamaica Bay Wildlife Refuge, Hempstead Bay, JFK and the NJ Meadowlands using mitochondrial DNA (Figure 4-1. A); I was also interested in determining whether a mitochondrial marker would possess the genetic signature of a bottleneck event due to the intense overharvesting of terrapins until the early 1900s

ii) To examine the relatedness of Diamondback terrapins in those urbanized areas to understand potential connections between terrapin populations; I was also interested in specifically examining the relationship between the terrapins in the Meadowlands and the other populations to understand the origin of the Meadowlands terrapins (Figure 4-1.B)

iii) To determine population parameters of the Meadowlands terrapins including population size, survival rates and recruitment rates.

I predicted a lack of genetic diversity, as indicated by the presence of very few unique mitochondrial DNA sequences, within terrapin populations due to the potential bottleneck through which the population passed (due to extensive harvesting in the past). Between populations, however, I predicted at least a moderate level of genetic diversity, as the literature indicates that terrapins do not disperse great distances. I predicted, therefore, that the Meadowlands population would be distinct from populations in New York, as they possibly expanded from a small number of turtles that were located in the Meadowlands in isolated pockets when human activity was intense. Isolation from the NY populations was probably achieved by the presence of the Hudson River, which terrapins are unlikely to cross because it represents unsuitable habitat (Russ Burke, pers. comm.). I predicted the possibility of some genetic relatedness between the Meadowlands population and the NY populations, due to random transportation of turtles by humans and occasional turtles able to make the migration.

With regards to the population characteristics of the Meadowlands, I predicted a fairly large population size and a possible effect of sex on survival probabilities. Females are subject to predation and road mortality when making nesting forays, so I predicted that females would have slightly lower survival probabilities than males.

4.2. Materials and Methods

4.2.1. Genetic Samples

Jamaica Bay and other NY locations: Blood samples for genetic analysis were taken during a nesting ecology study at Jamaica Bay Wildlife Refuge. Females were followed as they came ashore to nest; once they completed their nesting activities, they were captured and taken to a common processing area. Females that did not nest were captured before they returned to the water. Once blood was collected and information for the nesting ecology was recorded, terrapins were released from the common processing area, to return to the bay. Approximately 300 µl of blood was collected by inserting a small-gauge needle between caudal (tail) vertebrae into the caudal vein, and blood was blotted onto gauze. The gauze was then placed in an envelope and stored in a container of Drierite desiccant at room temperature. Similar procedures were used for the turtles in the Hempstead and JFK populations. The population at John F Kennedy National Airport was sampled during nesting season, as females came ashore in search of nesting sites. Females were collected by airport personnel as they (the turtles) attempted to cross runways. Terrapins from Hempstead Bay were collected by local NY Department of Environmental Conservation employees and were included in this study to understand movement of terrapins in the absence of a barrier to dispersal such as the Hudson River. Blood samples were collected June-July 2011.

A

B.

Figure 4-1. A. Location of NY sites for terrapin samples. B. NY sites in relation to the NJ Meadowlands site.

NJ Meadowlands: A NJ Meadowlands commission pontoon boat was used to set 12 baited Maryland-style crab traps at various locations throughout the Sawmill-Kingsland marsh system from 2009 to 2012 (Table 4-1, Figure 4-2.). Trap locations varied slightly from year to year depending on local conditions. Figure 4-3 shows trapping locations for 2011, which were representative of trapping locations throughout the study.

NJ Meadowlands Terrapin Trapping Days

2009	2010	2011	2012
22-Jul	11-Jun	8-Jun	12-Jun
30-Jul	18-Jun	23-Jun	26-Jun
25-Aug	25-Jun	6-Jul	12-Jul
	6-Jul	20-Jul	7-Aug
	20-Jul	11-Aug	
	17-Aug	24-Aug	
	26-Aug		
	16-Sep		

Table 4-1. Dates of trapping occasions in NJ Meadowlands.

Figure 4-2. Examples of Maryland style crab traps for the mark-recapture study in the NJ Meadowlands.

Figure 4-3. Trapping locations in the Sawmill Creek Wildlife Management Area in the NJ Meadowlands for 2011. Trapping locations for other years (2009, 2010, 2012) varied slightly.

Traps were placed in the water in the morning and retrieved between 2 and 5 hours later. Approximately 300 µl of blood was collected in June 2011 by inserting a small-gauge needle between caudal (tail) vertebrae into the caudal vein, and blood was blotted onto gauze. The gauze was then placed in an envelope and stored in a container of Drierite desiccant at room temperature. Both males and females were captured. Terrapins were tagged with a glass encapsulated 12.50 x 2.07 mm, 134.2 kHz Biomark Radio Frequency ID (RFID) Passive Integrated Transponder (PIT) tag, using a 12-gauge needle. Sex was recorded and measurements such as midline carapace length, midline plastron length, weight, scute number, maximum shell width, and maximum shell height were taken. Following data collection, a Biomark PIT tag pocket reader was used to verify the implanted tag number and then turtles were released at point of capture.

Total genomic DNA extraction was performed on the gauze via proteinase K digestion and silica spin column using a QIAmp DNA Mini Kit (QIAGEN) following the manufacturer's instructions, with the exception of ATL buffer and ProK amounts; double the amount of these two reagents were added. ProK digests were left overnight in a 56° C water bath and after approximately 24 hours, tubes were placed at -20° C for at least 2 days. This seemed to increase DNA yield.

For PCR, I used D-loop primers LGL283 and LGL1115 (Lamb and Osentoski 1997), which amplify a region of the mitochondrial control region, or D-loop approximately 500 bp in length. PCR reactions were carried out in a total volume of 25 µl, with

concentrations of reagents as follows: 1x PCR Buffer, 3.5 mM Magnesium Chloride, 0.2 mM dNTPs, 0.4 µM each of Forward and Reverse primer, and 0.1 units of *taq* polymerase. Ten (10) µl of genomic DNA was used. Conditions for amplification were based on Lamb and Osentoski (1997): 95° C initial denaturation for 5 minutes, 30 cycles of denaturation at 92° C for 1 minute, annealing at 50° C for 1 minute, extension at 72° C for 2 minutes and a final 7 minute extension at 72° C. Reactions were then held at 4° C.

PCR reactions were cleaned for sequencing using the silica based microcentrifuge protocol of the QIAquick PCR purification kit (QIAGEN). Thirty µl of buffer EB were added to more concentrated PCR products, while 20 µl were added to more dilute products. The same primers were used in sequencing reactions on an ABI 3130 Genetic Analyzer. The Big Dye terminator sequencing kit (Applied Biosystems) was used, with total reaction volumes of 20 µl. One µl of PCR product was used, with 5X sequencing buffer and a 10 µM concentration of primer. Parameters were as follows: 96° C for 1 min, 25 cycles of 96° C for 10 seconds, 50° C for 5 seconds, 60° C for 4 minutes, and a final 4° C hold. The oven temperature was preheated to 60° C while loading sequencing plates.

4.2.2. Estimates of Genetic Diversity

Sequences were initially aligned using MUSCLE (http://www.ebi.ac.uk/Tools/services/web/toolform.ebi?tool=muscle), to be used for statistical analyses. Sequences were checked for errors and trimmed to maximize the

cleanest regions of sequence, to obtain a final sequence of 362 base pairs. A statistical parsimony network was drawn using the method of Templeton (1992) via the program TCS (Clement *et al.* 2000). Estimates of genetic diversity within and among populations, including mean number of pairwise differences, average gene diversity, haplotype diversity, the number of polymorphic loci, and F_{st} were calculated using Arlequin v. 3.5 (Excoffier and Lischer 2010). Mismatch distributions were also obtained from Arlequin to test for a potential bottleneck signature within the mitochondrial D-loop. Essentially, the program plots the distribution of pairwise nucleotide site differences among different haplotypes and the resulting distribution can be related to particular demographic trends through a population's history. Stable populations have multimodal distributions (Rosenbaum *et al.* 2007; Markolf *et al*. 2008; Koumoundouros *et al.* 2009).

4.2.2. Demographic Information on Meadowlands Terrapins

Because of inconsistent time intervals between trapping days during each year, the encounter histories for each tagged terrapin were collapsed down into 4 occasions, one for each year. Maximum likelihood estimates of survival and recapture probabilities were generated using Cormack-Jolly-Seber (CJS) models in the program MARK (White and Burnham 1999). Candidate models were constructed to determine the effect of time and sex on survival and recapture and an information criterion-based approached (AICc) was used to determine the model that best fit the data (Table 4-2). The model with the lowest AICc value is the best model. Akaike weights can be used to determine the level of

confidence in a particular model; the higher the weight, the more evidence to support that model (Cooch and White 2010). With regards to the CJS models, since no one model obtained greater than 90% of the weight of evidence (AICc weight > 0.90), a model averaging approach was used.

Model	AICc	Delta AICc	AICc Weight	Model Likelihood	No. Par.
Phi(t)p(t*g)	681.1519	0.0000	0.32264	1.0000	8
Phi(t)p(t)	682.3734	1.2215	0.17517	0.5429	5
Phi(g)p(t*g)	682.7081	1.5562	0.1418	0.4593	8
Phi(g)p(t)	682.8173	1.6654	0.14031	0.4349	5
Phi(t*g)p(t*g)	684.3036	3.1517	0.06673	0.2068	10
Phi(.)p(g)	690.5250	9.3731	0.00297	0.0092	3
Phi(g)p(g)	690.5921	9.4402	0.00288	0.0089	4
Phi(g)p(.)	694.1927	13.0408	0.00048	0.0015	3
Phi(.)p(.)	694.8521	13.7002	0.00034	0.0011	2

Table 4-2. Candidate models for the Cormack-Jolly-Seber estimates of survival (Phi) and recapture (p) probabilities. Notation: “g” = group effect (sex), “t” = time effect, “t*g” = effect of interaction of time and sex.

In addition, the Jolly-Seber POPAN formulation was used to obtain estimates of probability of entrance (a metric that combines both recruitment and immigration), as well as population estimates (Table 4-3).

Model	AICc	Delta AICc	AICc Weight	Model Likelihood	No. Par.
p(g)phi(t)pent(t*g)	737.1207	0.0000	0.90538	1.0000	10
p(t*g)phi(t*g)pent(t*g)	743.3087	6.1880	0.04103	0.0453	14
p(.)phi(t)pent(t*g)	744.4694	7.3487	0.02297	0.0254	10
p(t)phi(t)pent(t*g)	745.4434	8.3227	0.01411	0.0156	11
p(t*g)phi(t)pent(t*g)	745.7317	8.6110	0.01222	0.0135	13
p(t)phi(t)pent(t)	748.1079	10.9872	0.00372	0.0041	9
p(g)phi(g)pent(t)	751.9189	14.7982	0.00055	0.0006	6
p(.)phi(.)pent(t*g)	758.9940	21.8733	0.00002	0.0000	8
p(t)phi(g)pent(t)	862.2254	125.1047	0.00000	0.0000	9
p(t*g)phi(g)pent(t*g)	864.7798	127.6591	0.00000	0.0000	14
p(g)phi(t)pent(t)	987.6777	250.5570	0.00000	0.0000	8

Table 4-3. Candidate models for the Jolly-Seber POPAN formulation. Notation: “g” = group effect (sex), “t” = time effect, “t*g” = effect of interaction of time and sex, “pent” = probability of entrance.

4.3. Results

4.3.1. Statistical Parsimony

According to TCS, there are 47 haplotypes, and only one haplotype is shared between two populations (the JFK9R haplotype from the JFK population is identical to the ML7JR haplotype from the Meadowlands). There is a significant level of haplotype diversity within the NY/NJ terrapins (Fig. 4-4). Sequences that were directly joined differed from each other by anywhere from between 1 to 14 nucleotides. While there does not seem to be any apparent clustering of the NY sequences, the Meadowlands sequences cluster among themselves more frequently than they cluster with other sequences.

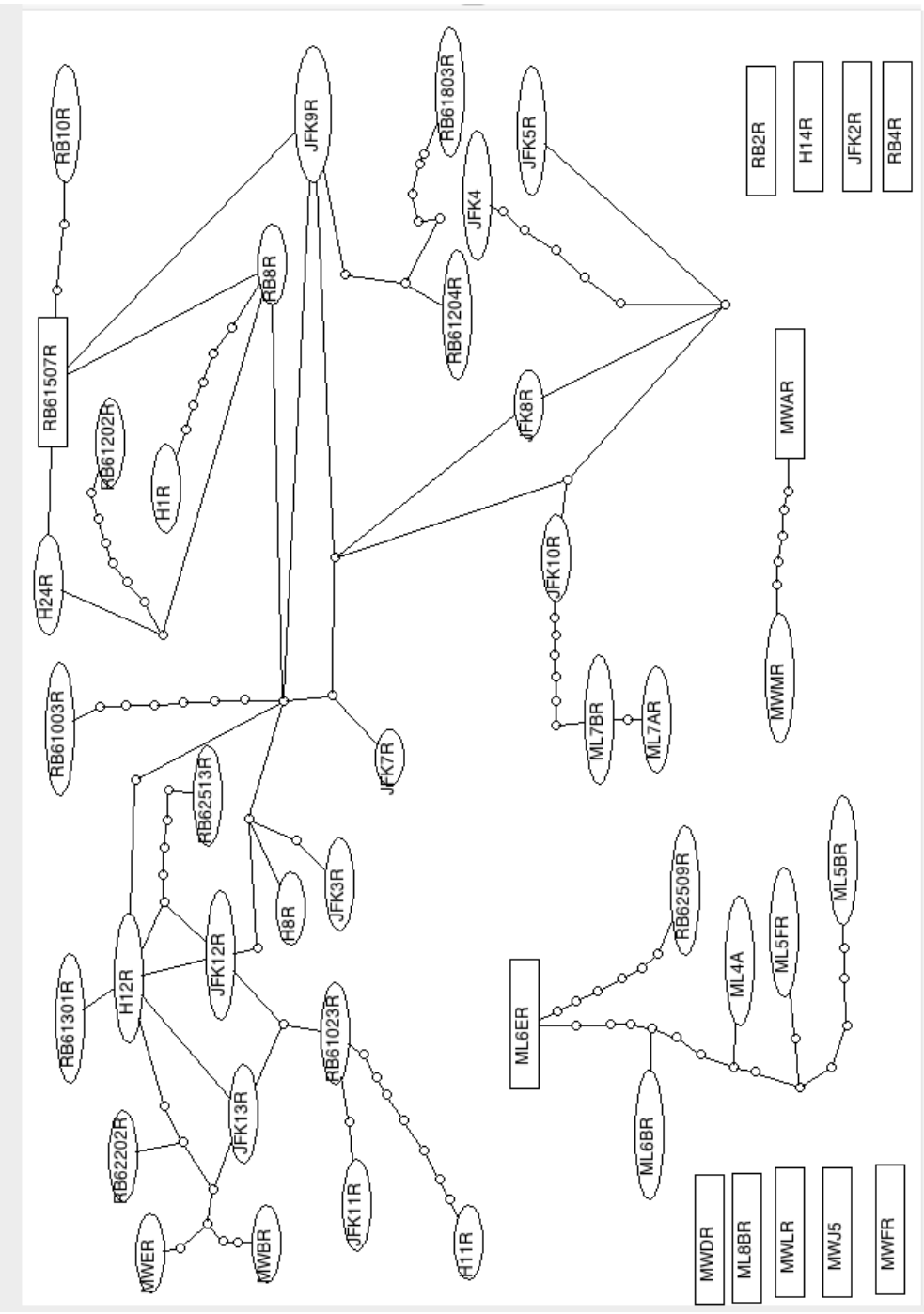


Figure 4-4. Statistical parsimony network generated by TCS. Notation = "RB" = Ruler's Bar, "JFK" = JFK airport, "H" = Hempstead, "ML" or "MW" = Meadowlands. Sequences RB2R, H14R, FK2R, RB4R, MWDR, ML8R, MWLR, MWJ5 and MWFR were not joined in the network by TCS with 95% certainty, suggesting they possess a greater number of nucleotide differences.

4.3.2. Estimates of Genetic Diversity using Arlequin

Average gene diversity for all populations pooled together was 0.9991 +/- 0.0045, number of polymorphic sites was 146, mean number of pairwise differences was 19.948582 +/- 8.975421 and average nucleotide diversity over all loci was 0.060268 +/- 0.030097. Theta S was 32.89797 (s. d. 9.44904) and theta pi was 19.94858 (s. d. 9.96221). I then separated the populations and computed the estimates of genetic diversity for the individual populations (Table 4-4).

Population	Number of polymorphic sites	Number of unique sequences	Nucleotide Diversity	Gene Diversity	Theta(S)	Theta(Pi)	Number of pairwise differences
Hempstead	88	6	0.096205 +/- 0.056774	1.000 +/- 0.0962	38.540146	31.26667	31.266667 +/- 15.979501
Meadowlands	110	17	0.078377 +/- 0.040540	1.000 +/- 0.0202	32.537361	24.845588	24.845588 +/- 11.483299
Ruler's Bar	39	14	0.028591 +/- 0.015685	1.000 +/- 0.270	12.263635	9.406593	9.406593 +/- 4.597160
JFK	14	11	0.014270 +/- 0.008534	1.000 +/- 0.0388	4.77984	4.709091	4.709091 +/- 2.496378

Table 4-4. Estimates of genetic diversity computed by Arlequin v. 3.5 for individual diamondback terrapin populations in NY and NJ.

Gene diversity indicates the probability of drawing the same two alleles at random from a sample of genes and is given by

$$1 - \sum p_i^2$$

Where p_i^2 is the probability of drawing the same allele twice

Gene diversity values range from 0 to 1. If a sample contains many alleles that are all present at similar frequencies, the gene diversity will be close to 1. If, instead, there are few alleles, with one being present at a significantly higher frequency than the rest, the value will be close to 0. Nucleotide diversity is a measure of the variation at individual nucleotide sites and in this case, the small number is indicative of the similarities between the sequences.

To assess genetic relatedness between populations, I obtained pairwise F_{st} values (Table 4-5). F_{st} is a measure of the degree of genetic differentiation between subpopulations and will vary from 0 to 1 (Beebee and Rowe 2007). The closer the value is to 1, the more genetically differentiated populations are, with a value of 1 indicating the populations are fixed for different alleles. The closer the value to 0, conversely, the less differentiated two populations are, with a value of 0 indicating no genetic differentiation at all. Little to no genetic differentiation means that there are either migrants being exchanged between the subpopulations OR that two subpopulations have split relatively recently in the past and not enough time has elapsed for them to accumulate differences (Templeton 1998,

Pearse and Crandall 2004). F_{st} values for the NY/NJ terrapins ranged from 0.06581 to 0.19473.

Population Pairwise Fsts

	Hempstead	Meadowlands	Ruler's Bar	JFK
Hempstead	0.00000			
Meadowlands	0.13266	0.00000		
Ruler's Bar	0.06581	0.19473	0.00000	
JFK	0.10822	0.18967	0.07489	0.00000

Table 4-5. Pairwise F_{st} values generated by Arlequin v. 3.5 to assess genetic differentiation among NY/NJ terrapin populations.

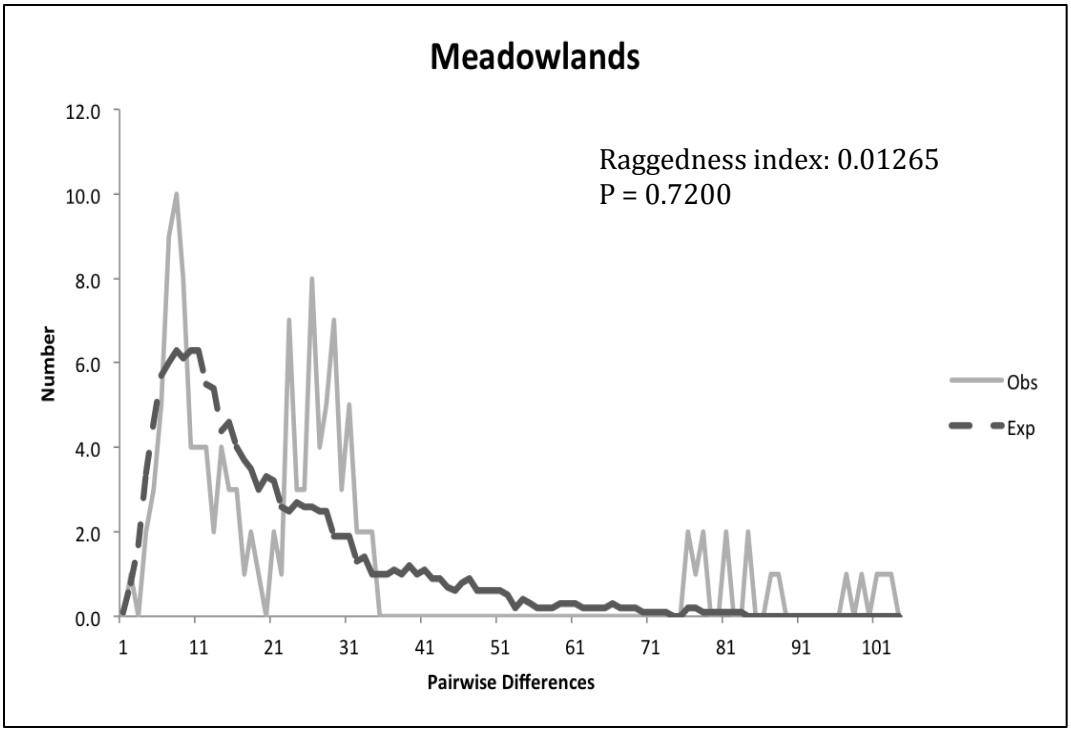
4.3.3. Mismatch Distributions

Diamondback terrapins most likely experienced a population bottleneck in the recent past, at the end of the 19th and beginning of the 20th centuries due to overharvesting. Indeed, terrapins grew more and more difficult to locate for the turtle soup market, despite high demand. In order to test for this bottleneck, I plotted the mismatch distribution output provided by Arlequin v. 3.5 (Figure 4-5, Figure 4-6, Figure 4-7). This provides a visual representation of the spread of nucleotide differences between pairs of

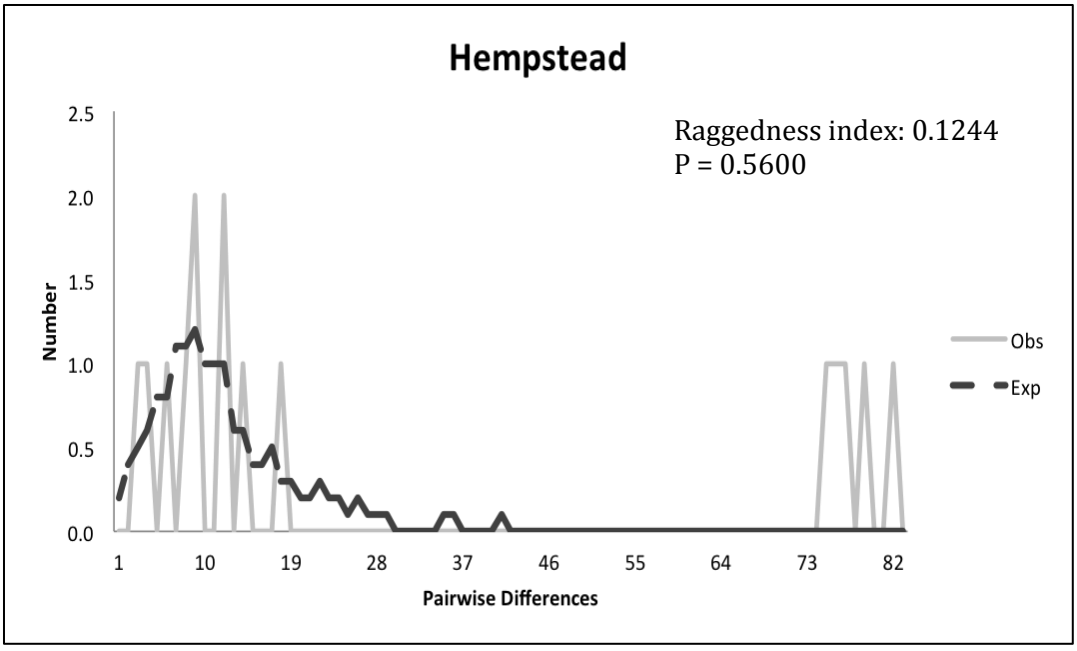
sequences (in this case, mitochondrial haplotypes). The shape of the distribution can give indications as to past population events. In particular, multimodal distributions are indicative of populations at stable equilbrium, unimodal distributions are indicative of populations that have undergone a recent range expansion and bimodal distributions are indicative of populations that have experienced a recent bottleneck (Johsnon *et al.* 2007, Markolf *et al.* 2008, Excoffier *et al*. 2009, Cooch and White 2010).

These distributions arise because of how mutations accumulate in DNA sequences. The oldest sequences in a population are going to differ by the largest number of mutations simply because the more time passes, the higher the probability of mutations occurring. As those older sequences give rise to younger sequences (moving forward in time to the present), those younger sequences will inherit the mutations of the ancestral sequences, but may also accumulate new mutations of their own. These younger sequences will have fewer mismatches, as they have had less time to accumluate a large number of differences (Wiley 2009).

Mismatch distributions are plotted against an expected null hypothesis of a recent range expansion, which produces a smooth unimodal curve. Raggedness indices, which are indicative of the fit between the observed mismatch distribution and this expected null model, are reported. P values indicate significance of deviation from the null model. I plotted mismatch distributions for each of the four populations separately, and then for all terrapin sequences pooled together.

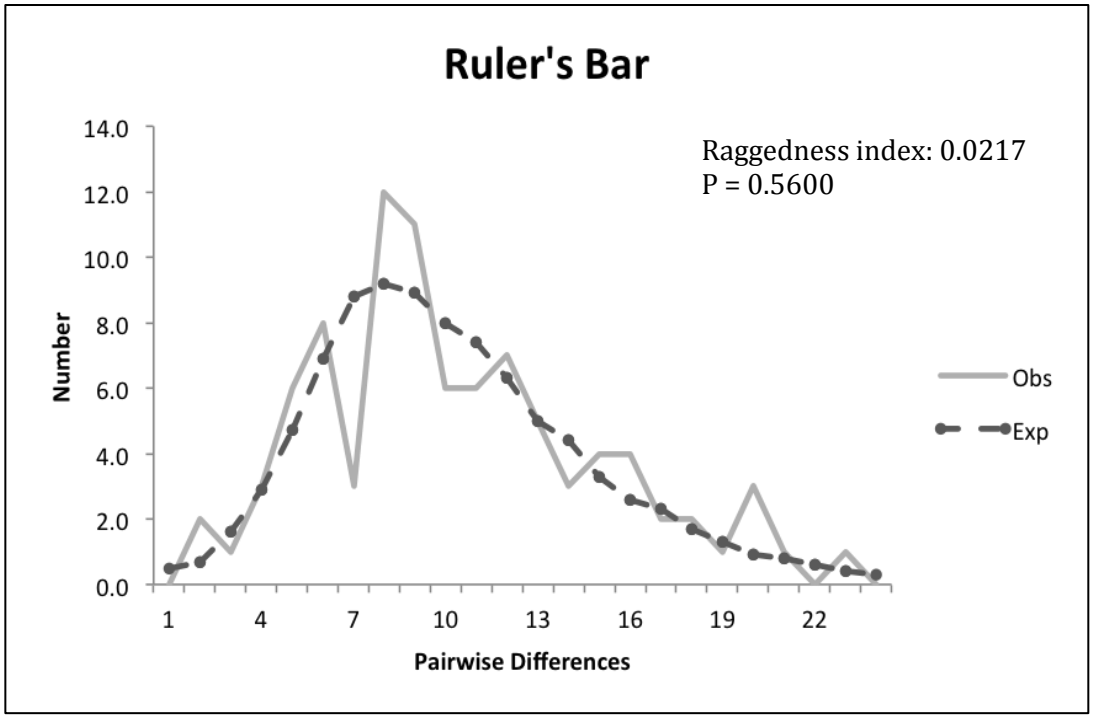


A.

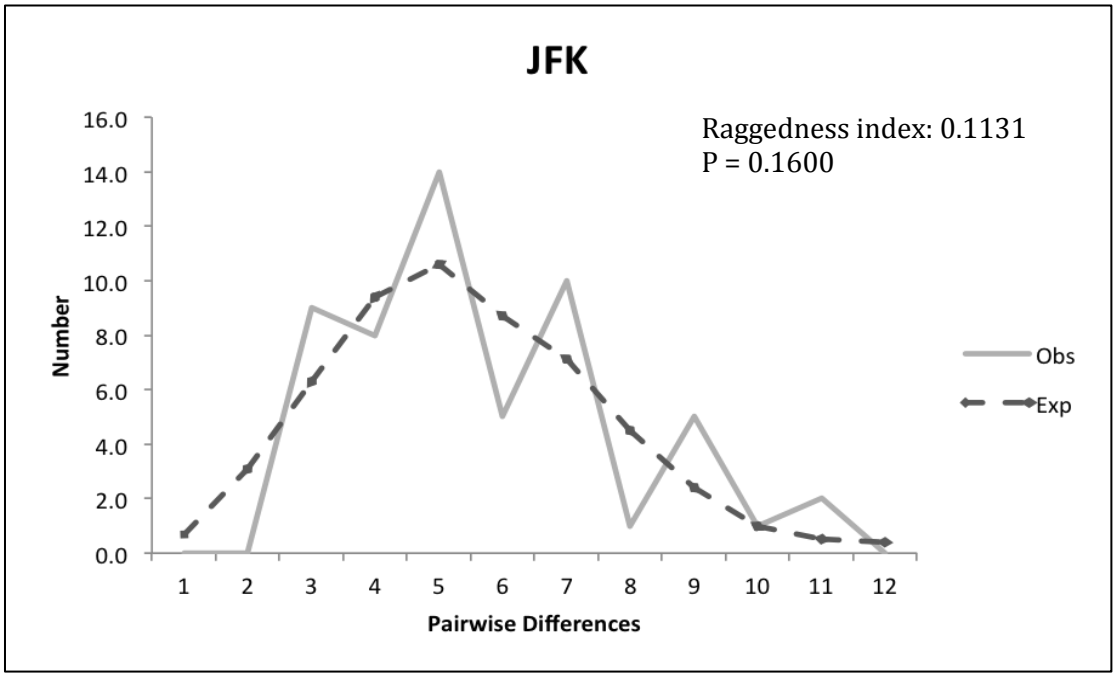


B.

Figure 4-5. Mismatch distributions for A. The NJ Meadowlands terrapin population and B. The Hempstead Bay terrapin population.



A.



B.

Figure 4-6. Mismatch distributions for A. The Ruler's Bar terrapin population and B. The JFK airport terrapin population (Jamaica Bay).

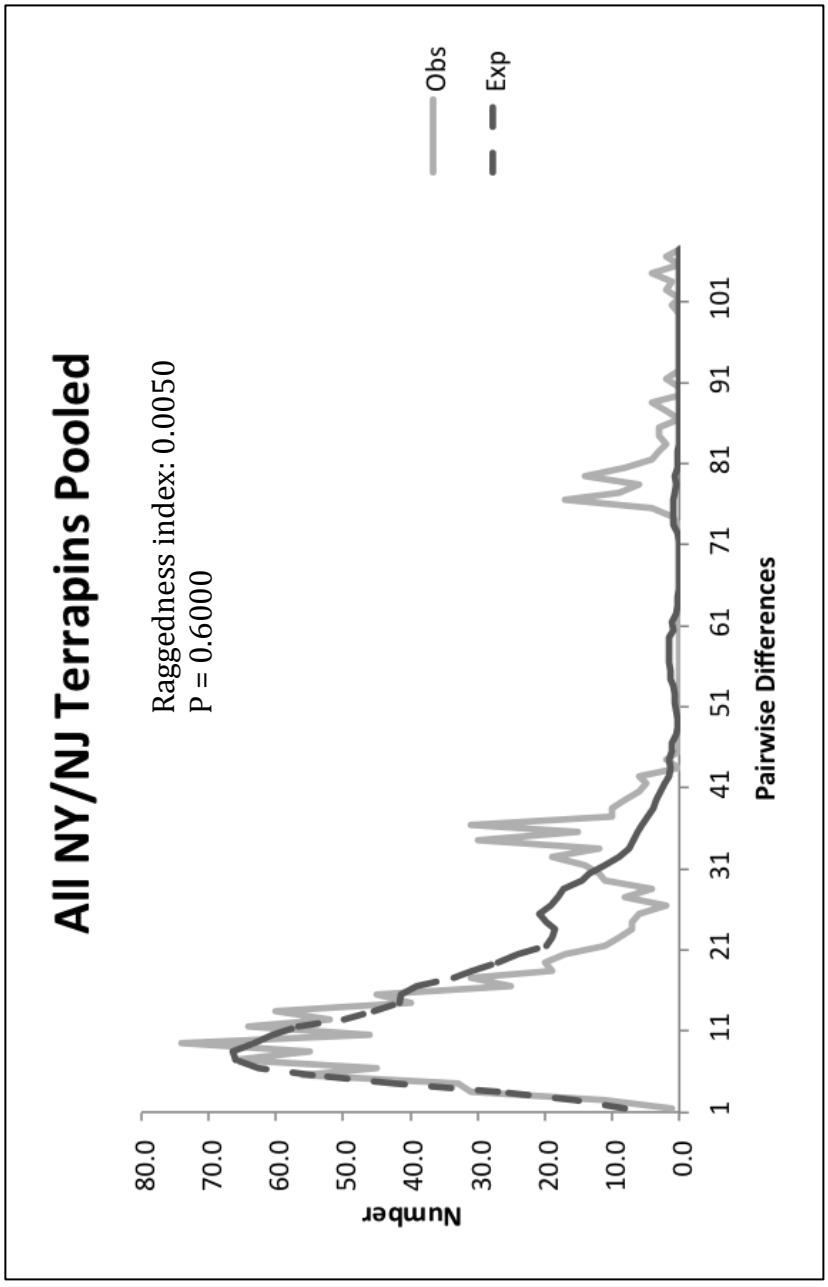


Figure 4-7. Mismatch distribution for all NY/NJ populations pooled together.

4.3.4. Demographic Parameters from Mark-Recapture Analysis

A total of 892 terrapins were marked between 2009 and 2012, with 418 female and 474 male. There were 141 recaptures. According to program MARK, the model with the most support out of the set of candidate models showed time dependence in survival probability and a combined effect of both time and sex for recapture probability. This model was approximately 1.84 times more likely than the next model, in which both survival and recapture probabilities showed time dependence and no effect of sex, and almost 2.3 times more likely than the next two models, which showed an effect of sex on survival and either a combined effect of sex and time on recapture or simply an effect of time. Because no one model received an AICc weight value of over 0.90 (indicating that it possessed at least 90% of the evidence to support it), a model averaging approach was implemented and estimates were obtained that took into account model uncertainty. Survival estimates were high for both males and females (0.933 for the first year of the study and 0.704 for the second year of the study for males; 0.943 for the first year of the study and 0.693 for the second year of the study for females). Recapture probabilities for males were 0.155 for the first year of the study and 0.161 for the second year of the study, while for females they were 0.207 and 0.243, respectively.

A Jolly-Seber POPAN formulation was used to estimate the population size of terrapins in the Meadowlands. This is an indication of the super-population from which the animals in the study could be drawn. The model that overwhelmingly received the

majority of support among the candidate model set was one which showed an effect of time on survival (with no effect of sex), an effect of sex on recapture probability and a combined effect of time and sex on the PENT parameter. PENT represents the "Probability of Entrance", which is a parameter that describes the likelihood of adding new individuals to the population; the PENT parameter includes the contribution of both immigration and recruitment. The number of males was estimated to be 1,770 while the number of females was estimated to be 1,377, for a total population of 3,147. According to the POPAN formulation, survival probability for the second year of the study was 0.668 and 0.404 for the third year of the study (for both males and females). Recapture probability was 0.140 for males and 0.233 for females. Finally, the Table 4-6 lists the PENT probabilities for males and females for the years of the study.

Probability of Entrance (PENT) paramer estimates

	Year 1 (2009-2010)	Year 2 (2010-2011)	Year 3 (2011-2012)
Males	0.127	0.485	0.114
Females	0.170	0.421	0.228

Table 4-6. Probability of Entrance parameter estimates from Jolly-Seber POPAN formulation.

4.4. Discussion and Conclusions

Reduced genetic diversity, which often accompanies a bottleneck event, can be detrimental to a population's long-term survival prospects. Lack of variation can deprive a population of the ability to respond to environmental change. Furthermore, a bottleneck event can increase the likelihood of inbreeding and this can lead to inbreeding depression (Keller and Waller 2002). For birds, this reduction in fitness could be manifested as a reduction in the number of hatchlings, a decreased fledgling rate or a decreased rate of recruitment to reproductive age (Schmoll *et al.* 2005). Sperm deformities, sterility and decreased courtship frequency also can result (Pusey and Wolf 1996). The cheetah, one of the most inbred animals known, has a number of genetic deformities, thought to be the result of inbreeding depression. Inbred spiders have smaller egg masses and lower juvenile survival (Pusey and Wolf 1996). A reduction of genetic diversity can also increase susceptibility to diseases and parasites; indeed, homozygous Soay sheep in an isolated, inbred population suffered higher parasite loads than the heterozygous members of the population (Keller and Waller 2002).

Diamondback terrapins were subjected to intense harvesting practices in the late 1800s and early 1900s and records indicate that populations throughout the range suffered drastic reductions (Roosenburg 1990). A drastic decrease in numbers of this nature often leaves a genetic signature. I used mitochondrial DNA to detect this signature. I plotted mismatch distributions of pairwise differences among DNA sequences and found that there was an approximately bimodal distribution when pooling all NY/NJ sequences

together, suggesting the presence of a bottleneck signature (Markolf *et al.* 2008). The very low raggedness index and high P value indicated, however, that there was a strong signal of recent population expansion in the data; indeed, low raggedness values are indicative of younger, expanding populations (Campos-Krauer and Wisely 2011, Ray *et al.* 2003). Supporting the concept of population contraction followed by a recent range expansion is the presence of high gene diversity and low nucleotide diversity. This indicates that there are a large number of different sequences that, however, only diverge on average at a small number of nucleotide sites; this indicates that these sequences are young (the older a sequence is, the more time it has had to accumulate mutations) (Gaubert *et al.* 2009).

In order to determine the contribution of the subpopulations to the picture of terrapin historical demographic process in NY/NJ, I plotted the mismatch distributions for each of the subpopulations separately. Both the Meadowlands and Hempstead populations show similar patterns to that demonstrated by the overall population, though the Hempstead population shows a higher raggedness index. This indicates a weaker signal of population expansion, and a stronger contribution of recent population contraction. This could indicate that the Hempstead population was comparatively small until relatively recently. In contrast, both the Ruler's Bar and JFK populations show a much stronger signal of prolonged population expansion with their unimodal distributions centered over a mid-range of pairwise differences. This difference in population histories is most likely due to the availability of suitable habitat for terrapins in these two locations over a much longer

time span compared to the Meadowlands and Hempstead populations, therefore giving the Ruler's Bar and JFK populations more stable conditions under which to expand their numbers.

These different signals can be explained by the fact that terrapins show a limited capacity for dispersal (Gibbons *et al.* 2001, Avissar 2006, Harden *et al.* 2007) and therefore different subpopulations will tend to diverge in their genetic characteristics. Indeed, when looking at pairwise F_{st} values, we see moderate to great genetic differentiation among and between NY/NJ subpopulations. Wright (1978) postulated that F_{st} values between 0.05 and 0.15 show moderate genetic differentiation, while values from 0.15 to 0.25 show great genetic differentiation. Using these benchmarks, the weakest signals are found between Ruler's Bar and Hempstead (0.066) and Ruler's Bar and JFK (0.075), and therefore among the NY populations. The strongest signals are found between the NY populations and the Meadowlands population (0.133 with Hempstead, 0.195 with Ruler's Bar and 0.190 with JFK). This corresponds with the idea that the Meadowlands terrapins have been isolated from the NY populations and that long-distance migrations are unlikely for this species. Besides unwillingness to cross areas of unsuitable habitat, lack of long-distance migration in terrapins may be due to this species' propensity for site fidelity. In particular, many females of various turtle species show nest site fidelity, and this behavior translates into clustering of mitochondrial haplotypes according to geographic location (Encalada *et al.* 1998, Bowen and Karl 2007, Carreras *et al.* 2007). This is precisely the pattern demonstrated in this study. The idea of site fidelity is further

evidenced by the moderate level of genetic differentiation between Ruler's Bar turtles and JFK turtles; there are no obvious barriers to movement between these two locations, so any genetic divergence is most likely caused by female turtle preference to nest in either one or the other location.

Finally, as mentioned, there is a significant level of genetic differentiation between the Meadowlands population and the NY populations. In terms of the origin of the Meadowlands population, this could indicate that the present-day population of Meadowlands terrapins resulted from the expansion of a small number of terrapins that persisted in isolated pockets of the Meadowlands, rather than from immigration from the NY populations. Their genetic history is also slightly different than the NY terrapins, having gone through a bottleneck event much more recently than either the Ruler's Bar or JFK populations, the more stable populations in NY. Hempstead provides an exception, most likely due to the more recent nature of that population. Results from the mark-recapture study add to this picture, showing a population that has grown to an estimated 3,147 individuals in the 37 years since terrapins were first mentioned in the NJ Meadowlands. Furthermore, survival rates of adults were high (which is not surprising for a long-lived turtle species). The lower recapture rates I found for males could be consistent with the idea that while females show high levels of site fidelity, males are the dispersers in this species.

In summary, we can see that Diamondback terrapins did experience a bottleneck, but seem to be recovering from this decrease in population size. Genetic diversity was reduced in the past, but populations in NY and NJ are expanding in such a way as to show new mutations in each of the populations studied. This expansion is occurring in highly urbanized habitats, which indicates that terrapins can thrive alongside human activity if properly managed. Female site fidelity could pose a problem if nesting locations are lost, as is occurring in Jamaica Bay due to the degradation of marshes. Terrapins there seem to be responding by switching nesting locations from Ruler's Bar to the JFK airport marsh, JoCo marsh; this is causing problems, though, as female terrapins are crossing airport runways in their search for nesting sites. If the Port Authority of New York and New Jersey chooses to exclude terrapins from JoCo marsh, the impact on the population of terrapins around JFK and Ruler's Bar could be significant. Terrapins in urban environments also face the threat of subsidized predators (Russ Burke, pers. comm.), or animals that benefit from increased resource availability resulting from the presence of humans (Boarman 1997, Gompper and Vanak 2008). On Ruler's Bar, approximately 95-98% of nests are predated by raccoons (Russ Burke, pers. comm.). This seems to be a problem in the Meadowlands as well (Brett Bragin, pers. comm), though it has yet to be quantified. Road mortality does not seem to pose a significant threat in any of the populations studied, as females do not need to cross any major roadways in order to find suitable nesting sites. Crab pot mortality is also virtually non-existent, as crabbing is not practiced in the locations under study. Therefore, if provided with sufficient nest

site locations and afforded protection from predation, then terrapins can fare well in areas impacted by anthropogenic activity.

CHAPTER 5

MANAGEMENT IMPLICATIONS

Herpetofauna are often limited in their ability to respond to anthropogenic disturbance mainly because of life history characteristics and limited dispersal capabilities. This means that an important survival strategy in a world of ever-increasing human development may be to live alongside humans, and tolerate a certain level of disturbance. This kind of co-existence may be successful only with appropriate management strategies, however. In order for these management strategies to be enacted, there must be an adequate understanding of the consequences of disturbance.

The presence of two emerging infectious diseases of amphibians, chytridiomycosis and Ranavirus, has now been documented in New Jersey. While chytridiomycosis does not seem to be a current threat, Ranavirus has been decimating certain amphibian populations. The locations in which it has currently caused mass mortality events are home to some of New Jersey's most sensitive herpetofaunal species, including Fowler's toads (*Anaxyrus fowleri*), the endangered Blue-spotted salamanders (*Ambystoma laterale*), the threatened Pine Barrens Tree frog (*Hyla andersonii*) and the threatened Northern Pine Snake (*Pituophis melanoleucus*). Little is known about Ranavirus' ecology and transmission in the wild, or its potential impact on species already in decline. Further

investigation of the extent of *Ranavirus* infection in New Jersey and its impact on both stable and declining species is critical in order to manage amphibian populations.

In particular, the finding of Ranavirus in New Jersey has led wildlife biologists and environmental educators to pay more attention to preventing the spread of the disease. There is evidence that both chytridiomycosis and Ranavirus have reached their global distribution mainly due to human activities. In parts of the United States, salamander larvae are sold as fish bait; studies have shown that many of these larvae are infected with Ranavirus (Picco *et al.* 2007, Schloegel *et al.* 2009). Ranavirus can also persist in wet sediment and water for up to two weeks (Daszak *et al.* 1999, Gray *et al.* 2009) and boots, vehicles and equipment that are not properly cleaned can transport virus particles in sediment and water between sites. In keeping with the directive issued by the World Organization for Animal Health (OIE) to take steps towards preventing the spread of Ranavirus and Bd, we have suggested that all personnel that visit wetland habitats have decontamination protocols in place.

Besides spreading disease, human activities have also led, albeit indirectly (perhaps), to the spread of new herpetological species through range expansions. A case in point is the American Green Tree frog expanding its range into New Jersey. There is strong evidence that this species has now established itself in southern NJ because of increasing temperatures and the creation of favorable environmental conditions. As of now no known negative consequences have resulted from the presence of *Hyla cinerea* in NJ. It

has demonstrated dietary plasticity outside of its native range (Leavitt and Fitzgerald 2009), but it does not seem to have impacted ecosystems in NJ. However, there is a concern. There have been documented cases of hybridization between *Hyla cinerea* and *Hyla andersonii*, the Pine Barrens tree frog, in Florida (Anderson and Moler 1986). New Jersey has a disjunct population of *H. andersonii* in southern NJ, in the vicinity of the locations that are being colonized by the green tree frog; the NJ population of *H. andersonii* is state threatened. Studies have shown that habitat degradation often leads to *H. cinerea* coming into contact and breeding with other hylid species, including *H. andersonii* (Anderson and Moler 1986, Aresco 1996). Hybrids showed no developmental abnormalities, but were infertile (Anderson and Moler 1986). Female *H. andersonii* seem to actually prefer male *H. cinerea* (Anderson and Moler 1986) when the latter called more loudly than males of their own species. This could potentially negatively impact the threatened population of Pine Barrens tree frogs by "contaminating" the gene pool. Close observation of this species in southern NJ is critical to prevent large-scale disruptions of local ecological communities. Future management strategies will benefit from the results of this monitoring.

Finally, there are examples of species successfully thriving in the face of anthropogenic disturbance, such as the Diamondback terrapins in Jamaica Bay, NY and the NJ Meadowlands. Understanding the characteristics of these populations can shed light on how to manage other urban-dwelling wildlife for both the benefit of ecosystem health and urban ecosystem. Fortunately, the appreciation for urban ecology is growing. Direct

exposure to the outdoors and wildlife has been shown to improve the well being of humans (Maller *et al.* 2005, Berman *et al.* 2012) and their understanding of the importance of environmental issues (Strife and Downey 2009). People who live in urbanized areas often have limited opportunities to get outdoors and are forced to seek ecological experiences close to home (Strife and Downey 2009). The establishment and maintenance of parks and refuges that are home to wildlife, like reptiles and amphibians, are extremely important for these individuals. Reptiles and amphibians are ideal for urban wildlife refuges, as they do not require vast open spaces for survival. An improved understanding of environmental issues on the part of people who live in urban areas can lead to increased support for conservation programs.

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APPENDIX A

Hyla cinerea ND1 Sequences

L1

TTCGTTTGTTCAACGATTAAAACCCTACGTGATCTGAGTTCAGACCGGAGCAATCCA

GGTCAGTTTCTATCTATAACAGCGCTTTCTCTAGTACGAAAGGACCGAAAAAGCATG

GTCCATGTATTATGCAAACCATACCCGATTATTTATGACCTAGTCTCAATAAACACTC

AACCTACTACCTCTCTCAAGACAAGAGTAGTTAATGTAGCAAAATCTGGTTTTGCAA

AAGACCT?AAACCCTTTCTATAGAGGTTCAAATCCTCTCATTAACTTTGAACCTAATC

CAACTCATTCTTCCCCTTTTATATATTGCCCCGATCCTTCTTGCAGTTGCCTTCCTCAC

CCTTATTGAACGCAAAGTGCTTGGCTATATACAACATCGCAAAGGCCCCAACGTAGT

CGGCCCCACAGGCCTTCTTCAACCAATCGCTGATGGAGTAAAACTCTTCATTAAAGA

ACCCATCCGACCATCAAATTCATCTC

D4

TTCGTTTGTTCAACGATTAAAACCCTACGTGATCTGAGTTCAGACCGGAGCAATCCA

GGTCAGTTTCTATCTATAACAGCGCTTTCTCTAGTACGAAAGGACCGAAAAAGCATG

GTCCATGTATTATACAAACCATACCTGATTATTTATGACTTAGTCTCAATAAACACTC

AACCTATTATTTCCCTCAAGACAAGAGCAGTTAATGTAGCAAAATCTGGTTTTGCAA

AAGACCT?AAACCCTTTCTATAGAGGTTCAAATCCTCTCATTAACTTTGAACCTAGTC

CAACTTATTCTCCCCCTTTTATATATTGCTCCGATCCTTCTTGCAGTTGCCTTCCTCAC

CCTTATTGAACGCAAAGTGCTTGGTTATATACAACACCGCAAAGGCCCCAACGTAGT

CGGCCCCACAGGCCTCCTTCAGCCAATCGCTGATGGGGTAAAACTTTTCATTAAAGA

ACCCATCCGACCATCAAATTCATCTC

DE1

TTCGTTTGTTCAACGATTAAAACCCTACGTGATCTGAGTTCAGACCGGAGCAATCCA

GGTCAGTTTCTATCTATAACAGCGCTTTCTCTAGTACGAAAGGACCGAAAAAGCATG

GTCCATGTATTATACAAACCATACCTGATTATTTATGACTTAGTCTCAATAAACACTC

AACCTATTATTTCCCTCAAGACAAGAGTAGTTAATGTAGCAAAATCTGGTTTTGCAA

AAGACCT?AAACCCTTTCTATAGAGGTTCAAATCCTCTCATTAACTTTGAACCTAGTC

CAACTTATTCTCCCCCTTTTATATATTGCTCCGATCCTTCTTGCAGTTGCCTTCCTCAC

CCTTATTGAACGCAAAGTGCTTGGTTATATACAACACCGCAAAGGCCCCAACGTAGT

CGGCCCCACAGGCCTCCTTCAGCCAATCGCTGATGGGGTAAAACTTTTCATTAAAGA

ACCCATCCGACCATCAAATTCATCTC

DE2

TTCGTTTGTTCAACGATTAAAACCCTACGTGATCTGAGTTCAGACCGGAGCAATCCA

GGTCAGTTTCTATCTATAACAGCGCTTTCTCTAGTACGAAAGGACCGAAAAAGCATG

GTCCATGTATTATACAAACCATACCTGATTATTTATGACTTAGTCTCAATAAACACTC

AACCTATTATTTCCCTCAAGACAAGAGTAGTTAATGTAGCAAAATCTGGTTTTGCAA

AAGACCT?AAACCCTTTCTATAGAGGTTCAAATCCTCTCATTAACTTTGAACCTAGTC

CAACTTATTCTCCCCCTTTTATATATTGCTCCGATCCTTCTTGCAGTTGCCTTCCTCAC

CCTTATTGAACGCAAAGTGCTTGGTTATATACAACACCGCAAAGGCCCCAACGTAGT

CGGCCCCACAGGCCTCCTTCAGCCAATCGCTGATGGGGTAAAACTTTTCATTAAAGA

ACCCATCCGACCATCAAATTCATCTC

DE3

TTCGTTTGTTCAACGATTAAAACCCTACGTGATCTGAGTTCAGACCGGAGCAATCCA

GGTCAGTTTCTATCTATAACAGCGCTTTCTCTAGTACGAAAGGACCGAAAAAGCATG

GTCCATGTATTATACAAACCATACCTGATTATTTATGACTTAGTCTCAATAAACACTC

AACCTATTATTTCCCTCAAGACAAGAGTAGTTAATGTAGCAAAATCTGGTTTTGCAA

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CAACTTATTCTCCCCCTTTTATATATTGCTCCGATCCTTCTTGCAGTTGCCTTCCTCAC

CCTTATTGAACGCAAAGTGCTTGGTTATATACAACACCGCAAAGGCCCCAACGTAGT

CGGCCCCACAGGCCTCCTTCAGCCAATCGCTGATGGGGTAAAACTTTTCATTAAAGA

ACCCATCCGACCATCAAATTCATCTC

DE4

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APPENDIX B

Malaclemys terrapin Dloop Sequences

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